



www.pearsoned.com

Group Art Unit: 1638

**Attorney**  
**Docket: 01/22171**  
**§**

**Attorney**

Docket: 01/22171

3

RECEIVED

AUG 18 2003

TECH CENTER 1600/2900

**DECLARATION OF PROF AARON KAPLAN UNDER 37 CFR 1.132**

I am presently employed as a full professor at the Hebrew University of Jerusalem, serving as the Chairman of the Department of Plant Science, where I am engaged in research and teaching. I also serve as the Director of the Minerva center for arid ecosystem research and a member of the Minerva Research Center on photosynthesis under stress.

I received my Ph.D. degree from the Hebrew University of Jerusalem in 1975, worked as a post-doctoral fellow in the Carnegie Institution at Stanford, California. During my post-doctoral studies, we discovered the CO<sub>2</sub> concentrating mechanism which operates in many photosynthetic microorganisms to raise the internal CO<sub>2</sub> concentration at the site of carboxylation. Over the years, I have served as a visiting Professor at Tokyo, Konstanz and Nagoya Universities. A Curriculum Vitae is appended.

My research focuses on Molecular mechanisms that drive ecological processes and on factors rate-limiting photosynthesis and growth of photosynthetic organisms. Since the beginning of my career, I have published over 100 scientific articles in highly regarded journals including PNAS, Current Biology, Plant Cell, JBC, Plant

Physiology and others, and have authored several invited reviews for, among others, *Annu. Rev. Plant Physiology Plant Mol. Biol.* Over the years I have presented my studies in many invited plenary, keynote and symposia lectures in international scientific conferences. I frequently serve as a reviewer for *Nature*, *Science*, *PNAS*, *JBC*, *Plant Cell*, *Plant Physiology* and others and for various granting agencies including NSF, USDA and DOE.

I am a member of several scientific societies including the American Society of Plant Biologists, The European society of Microbiologists and others.

I am a coinventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In a telephone interview of July 24, 2003, the Examiner requested clarification regarding the relevance of the reference cited in the previous communication of May 28, 2002 (Omata et al, *PNAS* 1999, 96:13571-76), from which the Examiner concluded that *cmpA* serve as the bicarbonate transporter in cyanobacteria and that the "enzymatic activity of the gene of SEQ ID NO:2 (*ictB* gene) remains in question". Such clarification is provided hereinbelow.

Firstly, regarding the role of bicarbonate transporters in  $\text{Ci}$  acquisition systems- I wish to point out that it is now well established that the ability to actively concentrate  $\text{CO}_2$ , against a gradient, results from the activity of at least 4 separate protein systems. This emerged from collaborative studies by Ogawa and myself : (Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., Kaplan, A. and T. Ogawa (2002) Genes Essential to Sodium-Dependent Bicarbonate Transport in Cyanobacteria: Function and Phylogenetic analysis. *JBC* 277: 18658-18664; and Shibata, M., Ohkawa, H., Kaneko, T., Fukuzawa, H., Tabata, S., Kaplan, A. and T. Ogawa (2001) Distinct constitutive and low- $\text{CO}_2$ -induced  $\text{CO}_2$  uptake systems in cyanobacteria: Novel genes involved and their phylogenetic relationship with homologous genes in other organisms. *Proc. Natl. Acad. Sci. USA* 98: 11789-11794).

Those were summarized in a recent invited review (Ogawa and Kaplan, *Photosynthesis Research*, in press, preprint enclosed). There are at least two  $\text{CO}_2$

uptake systems (an induced and a constitutive system), and two  $\text{HCO}_3^-$  transporter systems, the *cmpA-D* system, and the *SbtA* are active in cyanobacteria (see Table 2, page 4). These studies clearly indicated the limited role of the *cmpA-D* system, if any, in cyanobacteria since inactivation of this system in *Synechocystis* PCC 6803 (or *Synechococcus* PCC 7942) had little effect on either bicarbonate uptake or ability to grow under low  $\text{CO}_2$  conditions (see page 4, left column, paragraph 1).

Thus, it is evident that the *cmpA-D*  $\text{HCO}_3^-$  transporter system plays only a minor role in *Synechocystis* PCC 6803 growth, and that other, more important  $\text{HCO}_3^-$  transporter systems operate in this, and other, photosynthetic organisms. Indeed, we have recently shown that in a mutant of *Synechocystis* PCC 6803 where we inactivated  $\text{CO}_2$  uptake and  $\text{HCO}_3^-$  uptake by the *sbtA* system the ability to grow under low  $\text{CO}_2$  conditions was lost despite the fact that the *cmpA-D* operon was not affected. Inactivation of this operon in this mutant had no effect on growth (Shibata et al, J Biol Chem 2002, 277:18658-64, enclosed; see Figs. 1A and 1B).

In a very recent study, yet unpublished, we found that exposing a *Synechocystis* PCC 6803 mutant in which the two systems for  $\text{CO}_2$  uptake and *cmpA-D* and *sbtA* systems for  $\text{HCO}_3^-$  uptake were inactivated, to salinity restored  $\text{HCO}_3^-$  uptake capability and enabled the cells to grow under low  $\text{CO}_2$ , due to reactivation of the *ictB* system. This data provided additional evidence for the role of *ictB* in  $\text{HCO}_3^-$  uptake in cyanobacteria and for the presence of multiple pathways for  $\text{C}_i$  acquisition in these organisms, emphasizing the fallacy of Omata's conclusions.

Further, the Examiner has stated that "enzymatic activity of the gene of SEQ ID NO:2 (*ictB* gene) remains in question". It is my strong opinion that the bicarbonate transporter activity of the *ictB* gene product is amply demonstrated in the instant specification. For example, a *Synechococcus* PCC 7942 mutant where *ictB* was inactivated (mutant IL-2), was severely depressed in inorganic carbon uptake, compared with wild type (Figure 4a and 4b). The impaired  $\text{HCO}_3^-$  uptake was especially notable in low  $\text{CO}_2$  conditions (see Table 1, page 47). In another example, transgenic plants expressing the *ictB* gene demonstrated superior photosynthetic rate, compared to wild type plants, under conditions of limiting  $\text{CO}_2$  saturation, such as low humidity and low  $\text{CO}_2$  concentration (see Table 2, page 55). Further evidence of involvement of *ictB* gene in bicarbonate transport activity is provided by recent studies by myself, demonstrating enhancement of bicarbonate transport, resulting in increased

inorganic carbon fixation by transgenic tobacco plants expressing the *ictB* gene (see Figure 12, enclosed herein). Briefly, RubisCO activity was measured in wild type, and transgenic tobacco plants expressing the *ictB* gene, under conditions of low humidity (stomal closure, limited gas exchange), and thus, only partial activation of the enzyme complex by CO<sub>2</sub>. RubisCO activity was expressed as rate of carboxylation, directly measuring nmol CO<sub>2</sub> fixed per nmol active sites. The transgenic plants (open circles) clearly had superior carboxylation rates under non-activated conditions (open circles) than the wild type controls (open squares). That this superior inorganic carbon fixation was due to an increased availability of CO<sub>2</sub> substrate, and not to alteration of Rubisco catalytic properties, is demonstrated by the kinetics (S/V vs. S) plots in the inset: note the higher reaction rate (V max) but similar substrate affinity (K<sub>m</sub>) of the Rubisco activity in transgenic and wild type plants. Thus, the expression of the *ictB* gene in the transgenic tobacco plants resulted in increased CO<sub>2</sub> availability under conditions of HCO<sub>3</sub><sup>-</sup> transport dependent C<sub>i</sub> acquisition. In a recent paper describing the work on the transgenic plants [Lieman-Hurwitz, J., Rachmilevitch, S., Mittler, R., Marcus Y., and A. Kaplan (2003) Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO<sub>3</sub><sup>-</sup> accumulation in cyanobacteria. Plant Biotechnology J. 1: 43-50] we provide yet further clear evidence for the higher CO<sub>2</sub> concentration at the site of Rubisco in the transgenic plants which express *ictB*. The CO<sub>2</sub> compensation point was lower in the transgenic *Arabidopsis* and tobacco plants. This can only be attained if the internal CO<sub>2</sub> concentration is higher in the plants expressing *ictB*.

Thus, it is my strong opinion that the *ictB* gene disclosed in the instant specification clearly encodes "a polypeptide having a bicarbonate transporter activity", as recited in now amended independent claims 1 and 16.

In the official action, the Examiner states that the specification fails to provide guidance for a nucleic acid that hybridizes to SEQ ID NO:2 and that encodes a protein with inorganic carbon fixation activity, methods of using it, and plants thereby obtained. The Examiner further states that identifying nucleic acids functionally related to a given nucleic acid is highly unpredictable, and that a great many proteins have "inorganic carbon fixation activity", requiring "undue trial and error experimentation of one of ordinary skill in the art".

The Examiner has also stated that the specification fails to provide adequate description of the claimed invention, since the "claims are broadly drawn to a multitude of DNA molecules that hybridize to SEQ ID NO:2, or that comprise "any variation of a portion of any size of SEQ ID NO:2...and the specification only describes a nucleic acid from *Synechococcus* that comprises SEQ ID NO:2".

I wish to point out that the restrictions imposed by now amended independent claims 1 and 16, namely, a "...polynucleotide encoding a polypeptide...having an amino acid sequence at least 95% homologous to the sequence as set forth in SEQ ID NO:3..." and a "polynucleotide encoding a polypeptide having a bicarbonate transporter activity..." constitute clear criteria by which candidate polynucleotides can be screened. Indeed, using the methodology described in the instant specification and in the Response to Official Action filed by the Applicant on November 29, 2002, for identifying sequences homologous to the *ictB* coding sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:3), I have succeeded in identifying a number of highly conserved peptide domains (see hydropathy plots Fig 10a and 10b, enclosed herein) which are characteristic of the *ictB* protein and its homologues from other species (see Fig 11, amino acid sequence alignment, enclosed herein).

Briefly, the *IctB* protein from *Synechococcus* PCC 7942 and homologous protein *Synwh0268* from *Synechococcus* sp Strain WH 8102 were analyzed for characteristic transmembrane (hydrophobic) and hydrophilic domains using the TopPred program. Identification of proteins having significant homology, and alignment of the amino acid sequences was performed using the CLUSTALW multiple alignment program.

Thus, I believe that we have demonstrated that, provided the teachings of the present invention, one of ordinary skill in the art would be expected to be able to make and use the nucleic acid constructs and selection methods disclosed therein without undue experimentation, and with a reasonable expectation of success.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false

statements may jeopardize the validity of the application or any patent issued thereon.

July 28, 2003

A handwritten signature in cursive script, appearing to read "A. Kaplan", is written over a horizontal line.

Prof. Aaron Kaplan

**Encl.:****Professor Aaron Kaplan - Brief Curriculum Vitae**

Born in Israel, Dec. 12, 1945. Married, three children.

- 1963-1966 Military service
- 1975 - Ph.D., The Hebrew University of Jerusalem (HUJ).
- 1976 -1977 Post Doctorate with Drs. O. Bjorkman and JA Berry, Carnegie Institute, Stanford University, USA
- 1977 - 1980 Lecturer, Department of Plant Sciences, HUJ.
- 1980 - 1984 Senior Lecturer, Department of Plant Sciences, HUJ.
- 1984 - 1989 Associate Professor, Department of Plant Sciences, HUJ.
- 1982 - 1984 Secretary, Botanical Society of Israel.
- 1985 - 1988 Head, Biological studies, HUJ.
- 1989 - Professor of Botany, HUJ.
- 1990 - 1994 Chairman, Department of Plant Sciences, HUJ.
- 1992 - 1998 Member International Committee for Photosynthesis.
- 1994- 1997 Scientific Director of the Botanical Garden, Mount Scopus, HUJ.
- 1996- 1998 Chairman, Environmental studies, HUJ.
- 2000 - Chairman, Department of Plant Sciences, HUJ.

Director of The Minerva Center for the study of arid Ecosystems

Member of The Avron-Evenari Minerva Center for Photosynthetic Research.

**Publications 1999-**

- Kaplan, A., Reinhold, L. (1999) The CO<sub>2</sub>-concentrating mechanism of photosynthetic microorganisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 539-570.
- Hadas, O., Pinkas, R., Delphine, E., Vardi, A., Kaplan, A. Sukenik, A. 1999 Limnological and ecological aspects of *Aphanizomenon ovalisporum* bloom in Lake Kinneret, Israel. *J Plankton Research* 21: 1439-1453.
- Schatz, D., Eshkol, R., Kaplan, A., Hadas, O., Sukenik, A. 1999 Molecular monitoring of toxic cyanobacteria. *Arch. Hydrobiol. Spec. Issues Advanc. Limnol.* 55: 45-54.
- Vardi, A. Berman-Frank, I., Rozenberg, T., Hadas, O., Kaplan, A., Levine, A. (1999) Programmed Cell Death of the Bloom Forming Dinoflagellate *Peridinium gatunense* is Mediated by CO<sub>2</sub> Limitation and Oxidative Stress. *Curr. Biol.* 9: 1061-1064.
- Mittler, R. Merquiol, E. Hallak-Herr, E. Rachmilevitch, S. Kaplan, A. Cohen, M. (2001) Living under a "dormant" canopy: a molecular acclimation mechanism of the desert plant *Retama raetam*. *Plant J.* 25:407-416.
- Hihara, Y., Kamei, A., Kanehisa, M., Kaplan, A. Ikeuchi, M. (2001) DNA Microarray Analysis of cyanobacterial Gene Expression during Acclimation to High Light. *Plant Cell* 13:793-806.
- Hadas, O., Pinkas, R., Malinsky-Rushansky, N., Shalev-Alon, G., Delphine, E.,

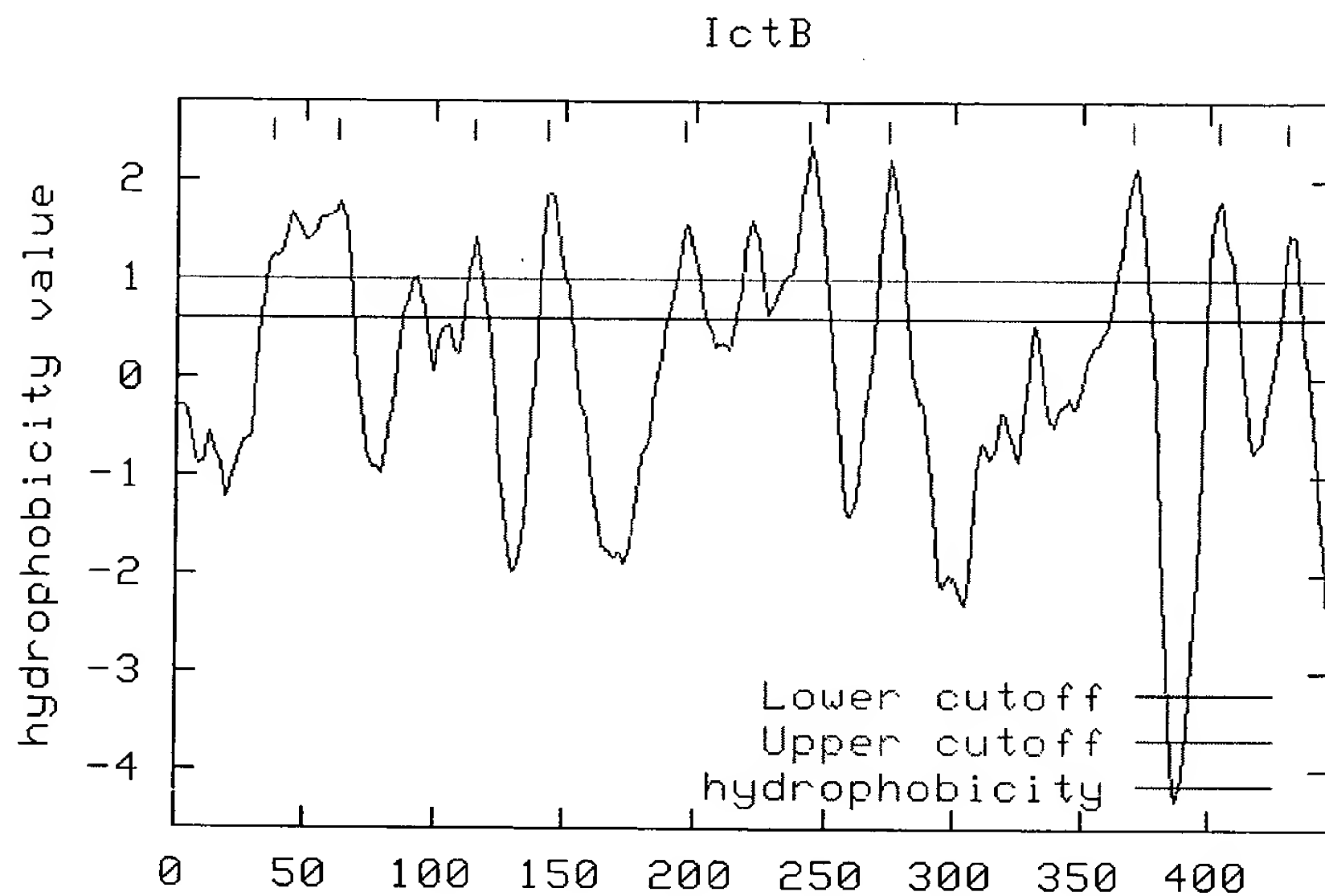
- Berner, T., Sukenik A. and A. Kaplan (2002) Can the physiological parameters determined in the laboratory explain the bloom of *Aphanizomenon ovalisporum* in Lake Kinneret? *Eur. J Phycology* 37: 259-267.
- Kaplan, A., Helman, Y., Tchernov, D. and Reinhold, L. (2001) Acclimation of photosynthetic microorganisms to changing ambient CO<sub>2</sub> concentration. *Proc. Natl. Acad. Sci. USA* 98: 4817-4818.
- Tchernov, D., Helman, Y., Keren, N., Luz, B., Ohad, I., Reinhold, L., Ogawa, T. and Kaplan, A. (2001) Passive entry of CO<sub>2</sub> and its energy-dependent intracellular conversion to HCO<sub>3</sub><sup>-</sup> in cyanobacteria are driven by a photosystem I-generated  $\Delta\mu\text{H}^+$ . *J. Biol Chem.* 276: 23450-23455.
- Shibata, M., Ohkawa, H., Kaneko, T., Fukuzawa, H., Tabata, S., Kaplan, A. and T. Ogawa (2001) Distinct constitutive and low-CO<sub>2</sub>-induced CO<sub>2</sub> uptake systems in cyanobacteria: Novel genes involved and their phylogenetic relationship with homologous genes in other organisms. *Proc. Natl. Acad. Sci. USA* 98: 11789-11794.
- Shalev-Alon, G., Sukenik, A., Livnah, O., R. Schwarz and A. Kaplan (2002) A novel gene encoding amidinotransferase in the cylindrospermopsin producing cyanobacterium *Aphanizomenon ovalisporum*. *FEMS Microbiol. Lett.* 209: 83-87.
- Merquiol, E., Pnueli, L., Cohen, M., Simovitch, M., Goloubinoff, P., Kaplan, A. and R. Mittler (2002) Seasonal and diurnal variations in gene expression in the desert legume *Retama raetam*. *Plant Cell Environ.* 25:1627-1638.
- Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., Kaplan, A. and T. Ogawa (2002) Genes Essential to Sodium-Dependent Bicarbonate Transport in Cyanobacteria: Function and Phylogenetic analysis. *JBC* 277: 18658-18664.
- Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002). Mechanism of dormancy and drought tolerance in the desert legume *Retama raetam*. *Plant J.* 31: 319-330.
- Sukenik, A., Eshkol, R., Livne, A., Hadas, O., Rom, M., Tchernov, D., Vardi, A., A. Kaplan (2002) Inhibition of growth and photosynthesis of the dinoflagellate *Peridinium gatunense* by *Microcystis* sp. (cyanobacteria): A novel allelopathic mechanism. *Limnology and Oceanography* 47: 1656-1663.
- Vardi, A., Schatz, D., Beeri, K., Motro, U., Sukenik, A., Levine, A. and A. Kaplan (2002) Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. *Current Biology* 12: 1767-1772.
- Tchernov, D., Silverman, J., Luz, B. and A. Kaplan (2003) Massive light-dependent cycling of inorganic carbon between photosynthetic microorganisms and their surroundings. *Photosynthesis Res.* (in press).
- Ogawa T. and A. Kaplan (2003) Inorganic carbon acquisition systems in cyanobacteria. *Photosynthesis Res.* (in press).
- Lieman-Hurwitz, J., Rachmilevitch, S., Mittler, R., Marcus Y., and A. Kaplan (2003) Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO<sub>3</sub><sup>-</sup> accumulation in cyanobacteria. *Plant Biotechnology J.* 1: 43-50.
- Helman, Y., Tchernov, D., Reinhold, L., Shibata, M., Ogawa, T., Schwarz, R., Ohad, I. and A. Kaplan (2003) Genes encoding A-type flavoproteins are essential for

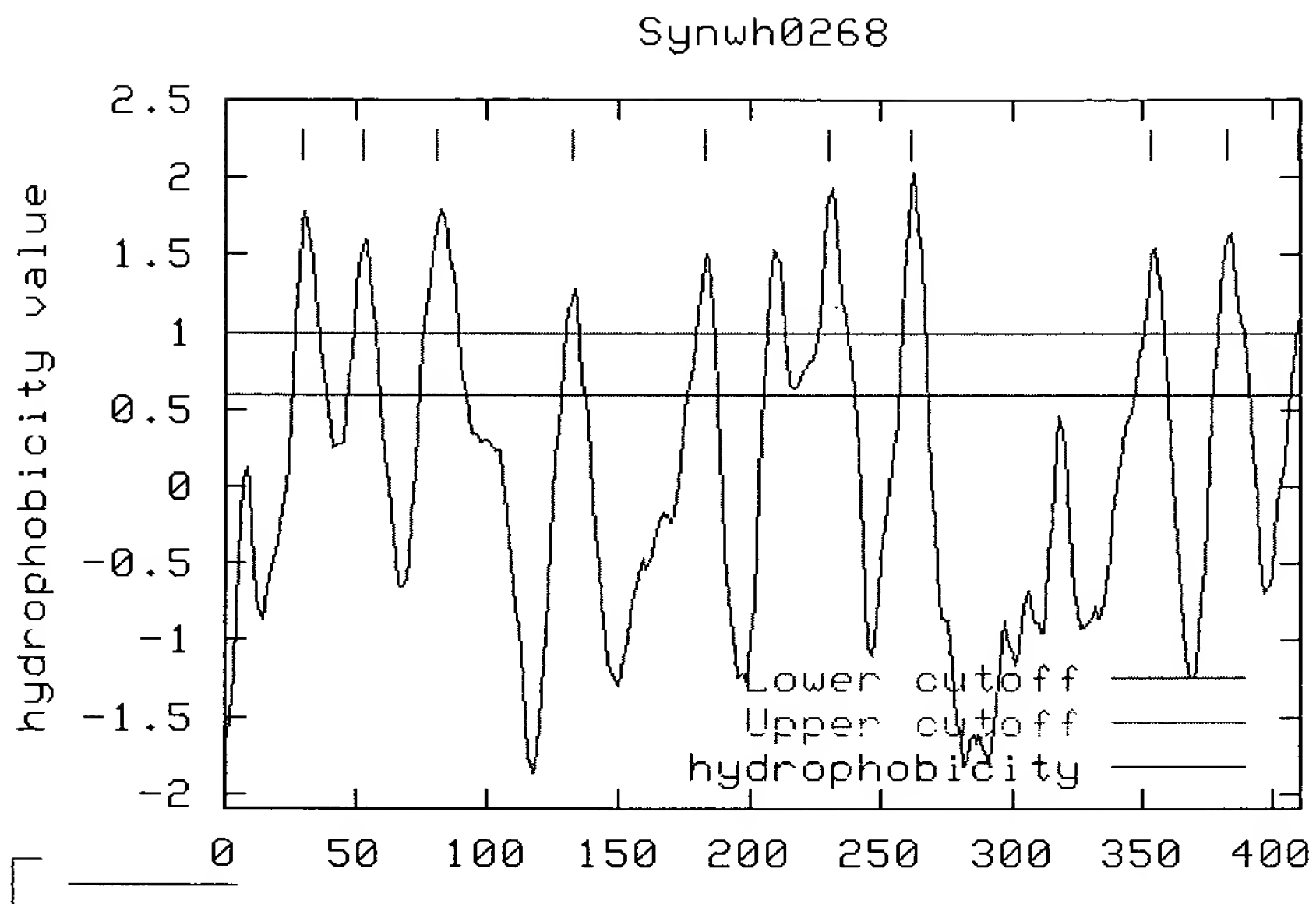


photoreduction of  $O_2$  in cyanobacteria *Current Biology* 13: 230-235.

**Figure 10: Hydropathy plots of two proteins, IctB from *Synechococcus* PCC 7942 and Synwh0268 from the marine *Synechococcus* sp. strain WH 8102.**

**10a**





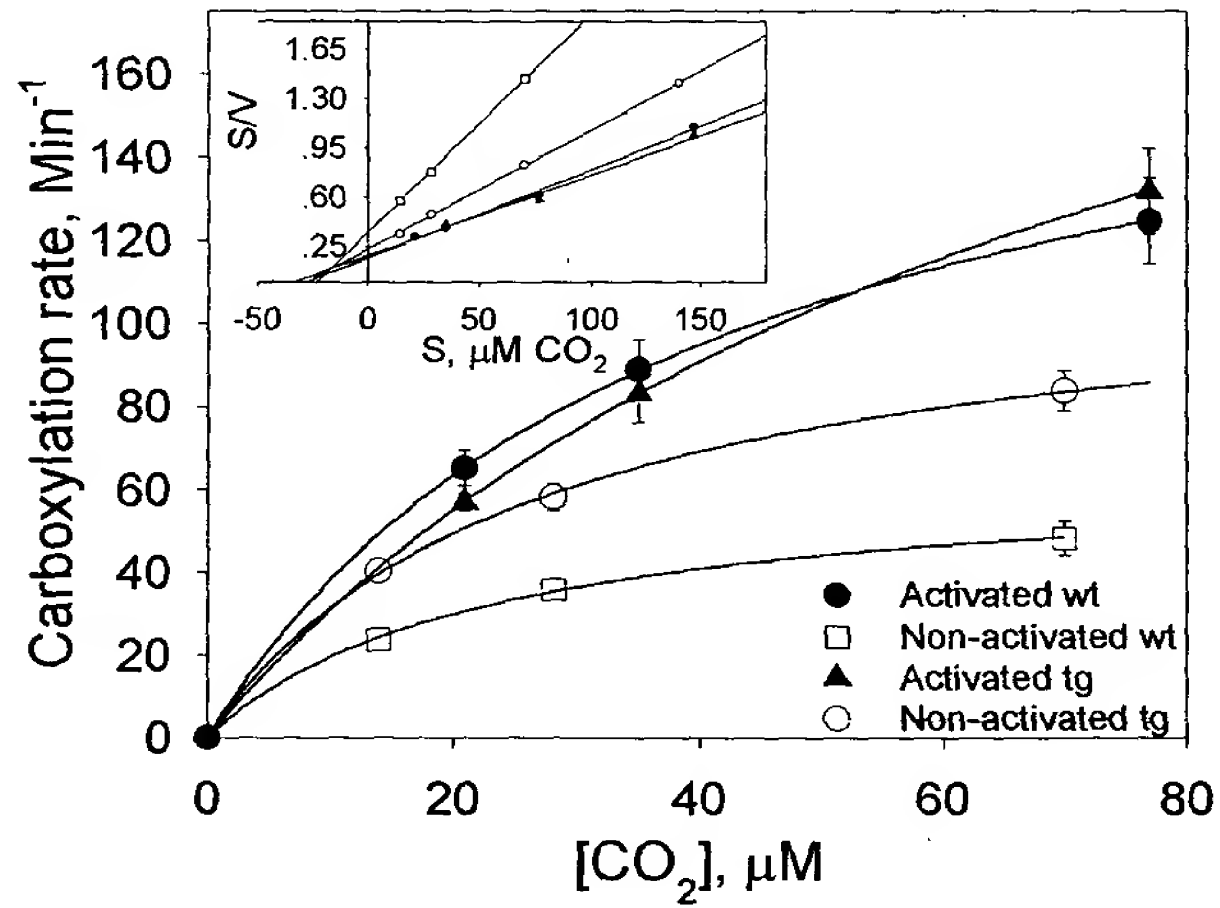
**10b**



Anabaena	GAPPLATWVDPESTLSKTTRVYSYLGNNPNLLAGYLVPAVIFSLMAVFVWQGWARKSLAVT						
Nostoc	GATALATWVDPESPLSKTTRVYSYLGNNPNLLAGYLLPAVIFSLVAIFAWQSWLKKALALT						
Trichodesmium	KVEPLATWNDPTSAQAGATRVYSYLGNNPNLLGGYLLPAIALSFVAIFAWSSWARKSLAVT						
SLR1515	GVEQLATWNDPTSTLAQATRVYSFGLGNNPNLLAAYLVPMTGLSLSALVVWRRWWPKLLGAT						
IctB	GVEELATWVDRNSVADFTSRVYSYLGNNPNLLAAYLVPTTAFSAAAIGVWRGWLPKLLAIA						
Thermosyn	GAEPLATWTDPESEALANVTRVYSFGLGNNPNLLAGYLLPSVPLSAAAIAVWQGWLPKLLAVV						
Prochloroco	PAEEMAHWADPNSVAAGTVRIYGPLGNNPNLLAGYLMPIPLALVALLRWQGLGAKLYAMV						
Synechococcus	STDELAGWADPNSVSAGTIRIYGPLGNNPNLLAGYLLPLVPLACI AVL RWKRLSCRLLAAV						
	. : * * * * . * : . * * * * . * : * : * : * : . . .						
		250	260	270	280	290	300
Anabaena	MLFVNTACLIFTYSRGGWIGLVVAVLGATALLVDWWSVQMPFFWRTWSLPILLGGLIGVL						
Nostoc	MLIVNTACLILTFSRGGWIGLVVAVLAVMALLVFWKSVEMPPFWRTWSLPILVGLGILIGIL						
Trichodesmium	ILLVSCACLRITGSRGWSWIGFLALMFAMLIIMWYWRSYMPSEFWQIWSLP IAVGSFAGLL						
SLR1515	MVIVNLLCLFFTQSRGGWLAVLALGATELALCYFWWLPQLPKFWQRWSLP LAIAVAVILG						
IctB	ATGASSLCLILTYSRGGWLG FVAMIFVWALLGLYWFQPRLPAPWRRWLPVVLGGLVAVL						
Thermosyn	MLGMNAASLILTFSRGGWLG LVAATIAGVLLGIWFWPRLPLQWRRWGVPTMGGLAIALC						
Prochloroco	ALGLGITATLFSFSRGGWLGMLSALAVILVLLLLRSTSHWPLVWRRLLPLIVIVLGTAML						
Synechococcus	TALLAGSATVFTYSRGGWLG LLAALALAGMLILLRTTAHWPLWRRLLPLAALLIAGIAL						
	. : * * * . * : . . . * * : * : * :						
		310	320	330	340	350	360
Anabaena	LIAVLFVEPVRFVLSIFADRQDSSNNFRNVWDAVFEMIRDRPIIGIGPGHNSFNKVYP						
Nostoc	LLAVIFVEPVRLRVFSIFADRQDSSNNFRNVWDAVFEMIRDRPIFGIGPGHNSFNKVYP						
Trichodesmium	ILAVVLEPLRDRVLSVFAGRQDSSNNFRMVWMSVFD MIRDRPILGIGPGNDVFNKIYP						
SLR1515	GGALIAVEPIRLRAMSIFAGREDSSNNFRINVWEGVKAMIRARPIIGIGPGNEAFNQIYP						
IctB	LVAVLGLEPLRVRVLSIFVGREDDSSNNFRINVWLAVLQMIQDRPWL GIGPGNTAFNLVYP						
Thermosyn	MGTIVSVPLRERAASIFVARGDSSNNFRINVWMAVQQMIWARPWL GIGPGNVAFNQIYP						
Prochloroco	VIAATQIEPIRTRITSLIAGRSDSSNNFRINVWLSSEMIQARPWL GIGPGNAAFNRIYP						
Synechococcus	ALAITQLDPIRTRVLSLVAGRGDSSNNFRINVWLAAIEMVQDRPWL GIGPGNAAFNNSIYP						
	: : * : * * : . . * * * * * * * * . * : * * : * * * : * * : * *						
		370	380	390	400	410	420
Anabaena	LYQR-PRYSALSAYSIFLEVAVEMGFVGLACFLWLIIVTINTAFVQLRQLRQSANVQGF						
Nostoc	LYQH-PRYTALSAYSILFEVTVETGFVGLACFLWLIIVTFNTALLQVRRLRLRSVEGE						
Trichodesmium	LYQR-PRYSALSSYSVPLEIVVETGFIGLTAFLWLLLVTFNQGVQLKRLRDADNPQGYW						
SLR1515	YYMR-PRFTALSAYSISYLEILVETGVVGFTCMLWLLAVTLGKGVELVKRCRQTLAPEGIW						
IctB	LYQQ-ARFTALSAYSVPLEVAVEGGLGLTAFWLLLVTAVTAVRQVSRLRRDRNPQAFW						
Thermosyn	LYQVNVRF TALGAYSIFLEILVEVGFIGFGVFLWLLAVLGD RARRCFEELRATGSPQGF						
Prochloroco	LFQQ-PKFNALSAYSVPLEILVETGLAGLMASLALVITGMRKGLAGLNSNHPL----ALP						
Synechococcus	LYQQ-PKFDALSAYSVPLEILVETGIPGLLACLGLLLSSIQRGLR-IHGQQ-----GLI						
	: : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *						
		430	440	450	460	470	480
Anabaena	LVGALATLLGMLAHGTVDTIWFRPEVNTLWWMVALIASYWTPLSANQCQELNLFKEEPT						
Nostoc	LIGAIAILLGMLAHGTVDTVWYRPEVNTLWWMVALIASYWTPLTQNQTNPS---NPEPA						
Trichodesmium	LIGAIAAMVGLIGHGLVDTVWYRPQVNTIWWLMVAIIASYSQQGVRSRE-----						
SLR1515	IMGALAAIIGLLVHGMVDTVWYRPPVSTLWWMVAIVASQWASQAARLEASKEENEDKPL						
IctB	LMASLAGLAGMLGHGLFDTVLYRPEASTLWWMLCIGAIASFQWQPQPSKQLPPEAEHSDEKM						
Thermosyn	LMGTIAAMIGMLTHGLVDTIWFRPEVATLWWMVAIVASFQWQPQPSKQKTANGTFSNRDPEP-						
Prochloroco	ALASLAAIAGLAVHGITDTIFFRPEVQLVGWFECLATLAQTQPEQKQLQOTE-----						
Synechococcus	AIGSLAAIAGLLTQGITDTIFFRPEVQLIGWFALASLGATWLRD-----						
	: : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *						

Anabaena	SN-
Nostoc	VN-
Trichodesmium	---
SLR1515	LAS
IctB	---
Thermosyn	---
Prochloroco	---
Synechococcus	---

Figure 12: RubisCO activity *in vivo* (non-activated) and *in vitro* (activated) in wild type (wt) and transgenic (tg) tobacco plant in low humidity as a function of  $[\text{CO}_2]$ . Inset: kinetics (S/V vs. S) plots of RubisCO activity. n=6.



results of **BLAST**

BLASTN 2.2.6 [Apr-09-2003]

**Reference:**

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Sch?ffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1056873405-010515-31569

**Query=**

(946 letters)

**Database:** All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

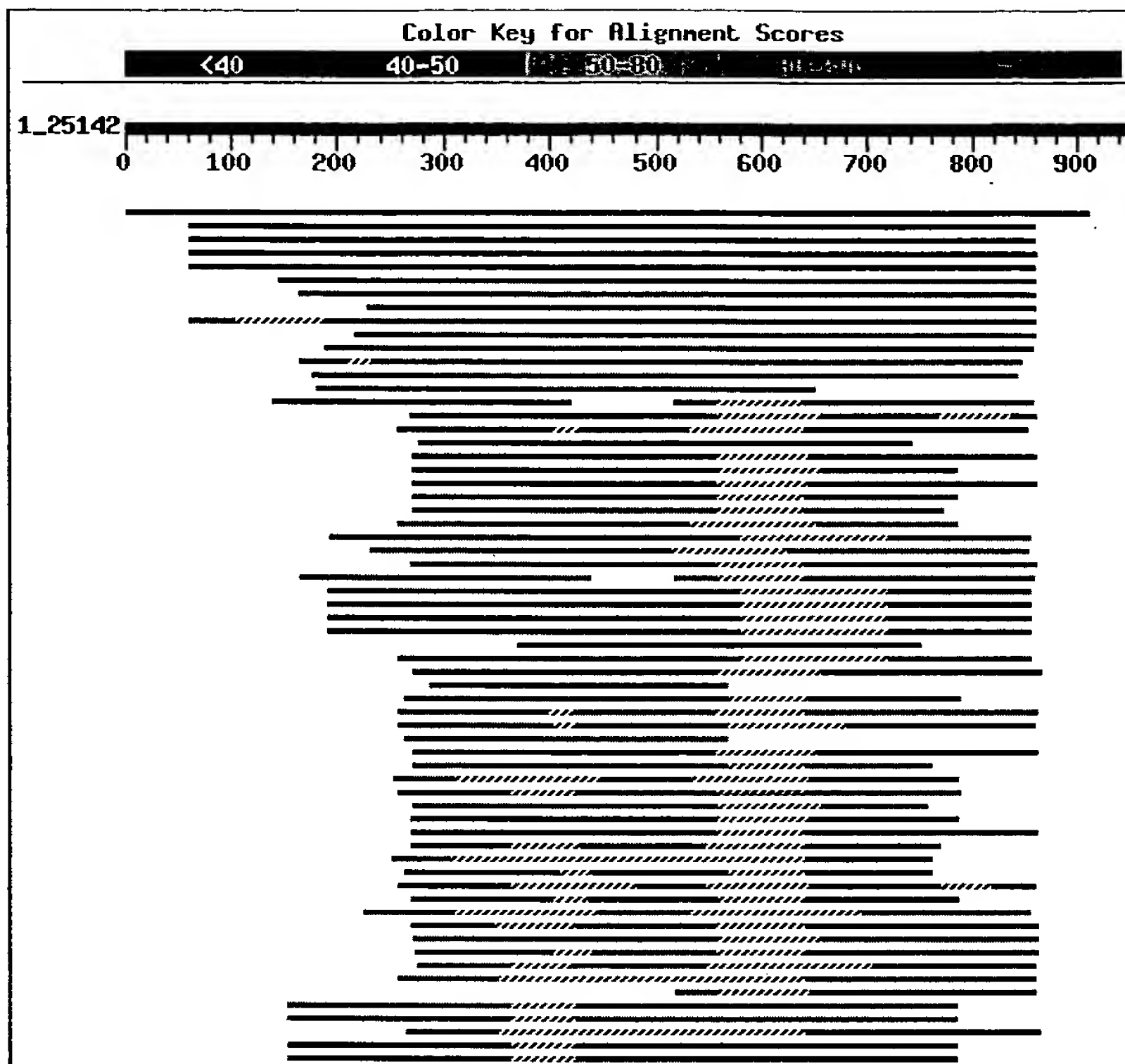
1,810,334 sequences; 8,500,654,645 total letters

If you have any problems or questions with the results of this search please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)**Distribution of 224 Blast Hits on the Query Sequence**

Mouse-over to show define and scores. Click to show alignments





Sequences producing significant alignments:		Score (bits)	E Value	
<u>gi 29123377 gb AY219853.1 </u>	Nicotiana tabacum chlorophyll a/...	1715	0.0	
<u>gi 170389 gb M17558.1 TOMCAB4A</u>	Tomato Cab-4 gene encoding c...	783	0.0	<b>U</b>
<u>gi 20511 emb X04966.1 PHCAB37</u>	Petunia Cab gene for chloroph...	638	e-180	
<u>gi 19826 emb X58230.1 NTCAB36</u>	Tobacco CAB36 gene for chloro...	617	e-173	
<u>gi 511152 emb Z35160.1 STCHLABP</u>	S.tuberosum gene for chloro...	607	e-170	
<u>gi 170391 gb M17559.1 TOMCAB5A</u>	Tomato Cab-5 gene encoding c...	593	e-166	<b>U</b>
<u>gi 3560528 gb AF039598.1 AF039598</u>	Prunus persica light harv...	464	e-127	
<u>gi 2765355 emb Y13865.1 BVCHLOROP</u>	Beta vulgaris mRNA for ch...	436	e-119	
<u>gi 398598 emb X74732.1 AHLHAH</u>	A.hypochondriacus Lhcb2*Ah1 mRNA	408	e-111	
<u>gi 18481 emb X54090.1 GHCAB</u>	G.hirsutum cab gene for chlorop...	371	1e-99	
<u>gi 5714655 gb AF165529.1 AF165529</u>	Rumex palustris chlorophy...	280	4e-72	
<u>gi 16805331 gb M97171.1 SOYCAB6A</u>	Glycine max chlorophyll a/...	272	1e-69	
<u>gi 9587202 gb AF279248.1 AF279248</u>	Vigna radiata LHCII type ...	232	8e-58	
<u>gi 169885 gb M16887.1 SIPCAB</u>	White campion chlorophyll a/b-...	224	2e-55	
<u>gi 16225449 gb AF417304.1 AF417304</u>	Castanea sativa putative...	216	5e-53	
<u>gi 20486 emb X02356.1 PECAB91R</u>	Petunia gene for chlorophyll...	170	3e-39	
<u>gi 2804571 dbj AB006081.1 </u>	Fagus crenata mRNA for chlorophy...	168	1e-38	
<u>gi 12240088 gb AF312227.1 AF312227</u>	Citrus reticulata light-...	167	4e-38	
<u>gi 19818 emb X52741.1 NTCAB16</u>	Tabacco Cab16 mRNA for major ...	165	2e-37	
<u>gi 3036954 dbj AB012641.1 </u>	Nicotiana sylvestris Lhcb1*9 gen...	159	1e-35	
<u>gi 19836 emb X58229.1 NTCAB7</u>	Tobacco CAB7 gene for chloroph...	157	4e-35	
<u>gi 3036952 dbj AB012640.1 </u>	Nicotiana sylvestris Lhcb1*8 gen...	157	4e-35	
<u>gi 170211 gb M21398.1 TOBCABB</u>	Tobacco chlorophyll a/b-bindi...	157	4e-35	

gi 2645998 gb AF034631.1 AF034631	Panax ginseng chlorophyll...	151	2e-33	
gi 21407315 gb AY088541.1	Arabidopsis thaliana clone 7700 ...	147	4e-32	U
gi 19822 emb X52743.1 NTCAB21	Tabacco Cab21 mRNA for major ...	145	2e-31	
gi 20657 emb X57082.1 PSCABII	P.sativum Cab II gene for chl...	145	2e-31	
gi 30688889 ref NM 113685.2	Arabidopsis thaliana light har...	143	6e-31	U
gi 30023745 gb BT006298.1	Arabidopsis thaliana At3g27700 m...	143	6e-31	U
gi 4741949 gb AF134125.1	Arabidopsis thaliana Lhcb2 protei...	143	6e-31	U
gi 22003725 gb AF526508.1	Vicia faba A-B binding protein (...)	143	6e-31	
gi 13899124 gb AF370557.1 AF370557	Arabidopsis thaliana lig...	143	6e-31	U
gi 454249 emb Z29965.1 TRLHCABBP	T.repens (Huia) mRNA for l...	143	6e-31	
gi 20482 emb X02358.1 PECAB25	Petunia gene for chlorophyll ...	141	2e-30	
gi 3702732 dbj AB018114.1	Arabidopsis thaliana genomic DNA...	141	2e-30	
gi 13676405 dbj AB050125.1	Amaranthus tricolor cab1b mRNA ...	137	4e-29	
gi 18551 emb X12981.1 GMCAB3	Soybean Cab3 gene for PSII LHC...	135	1e-28	
gi 14239 emb X14341.1 SOCABP	S.oleracea chloroplast mRNA fo...	133	6e-28	
gi 974849 emb X89023.1 HVLHCIITI	H.vulgare mRNA for LHC II ...	131	2e-27	U
gi 2196769 gb AF003127.1 AF003127	Mesembryanthemum crystall...	131	2e-27	
gi 3036947 dbj AB012638.1	Nicotiana sylvestris Lhcb1*5, Lh...	127	4e-26	
gi 170673 gb M10144.1 WHTCAB	Wheat major chlorophyll a/b-bi...	127	4e-26	
gi 6644195 gb AF207690.1 AF207690	Daucus carota cultivar Ku...	125	1e-25	
gi 2196773 gb AF003129.1 AF003129	Mesembryanthemum crystall...	125	1e-25	
gi 170209 gb M21397.1 TOBCABA	Tobacco chlorophyll a/b-bindi...	125	1e-25	
gi 170401 gb M14443.1 TOMCBPA	Tomato chlorophyll a/b-bindin...	123	6e-25	
gi 17736840 dbj AP004473.1	Lotus japonicus genomic DNA, ch...	121	2e-24	
gi 3036943 dbj AB012637.1	Nicotiana sylvestris Lhcb1*2, Lh...	121	2e-24	
gi 1053215 gb U39475.1 GMU39475	Glycine max chlorophyll a/b...	119	9e-24	U
gi 170423 gb M30622.1 TOMCBPF2	Tomato chlorophyll a/b-bindi...	119	9e-24	
gi 170418 gb M30620.1 TOMCBPE2	Tomato chlorophyll a/b-bindi...	119	9e-24	
gi 405616 emb X61610.1 BNLHCB3C	B.napus gene for LHCI Type...	119	9e-24	
gi 9587206 gb AF279250.1 AF279250	Vigna radiata LHCI type ...	117	3e-23	
gi 693919 gb U21113.1 STU21113	Solanum tuberosum chlorophyl...	117	3e-23	
gi 25702105 gb AC126013.9	Medicago truncatula clone mth2-3...	115	1e-22	
gi 3928139 emb AJ131044.1 CAR131044	Cicer arietinum mRNA fo...	113	5e-22	
gi 19828 emb X52744.1 NTCAB40	Tabacco Cab40 mRNA for major ...	113	5e-22	
gi 3294334 dbj AB012636.1	Nicotiana sylvestris Lhcb1*1 gen...	113	5e-22	
gi 289919 gb L07119.1 COTIIABINA	Gossypium hirsutum chlorop...	112	5e-22	
gi 18555 emb X16535.1 GMCAB4	G.max DNA for Cab4	111	2e-21	
gi 8954290 gb AF139465.2 AF139465	Vigna radiata LHCI type ...	111	2e-21	
gi 170411 gb M30618.1 TOMCBPD2	Tomato chlorophyll a/b-bindi...	111	2e-21	
gi 169200 gb K00974.1 PETCAB4	Petunia major chlorophyll a/b...	109	8e-21	
gi 3293554 gb AF072931.1 AF072931	Medicago sativa chlorophy...	109	8e-21	
gi 170427 gb M14449.1 TOMCBPG	Tomato chlorophyll a/b-bindin...	109	8e-21	
gi 170406 gb M30616.1 TOMCBPC2	Tomato chlorophyll a/b-bindi...	109	8e-21	
gi 169932 gb M21396.1 SOYCBPA	Soybean light-harvesting chlo...	109	8e-21	U
gi 167522 gb M16057.1 CUSLHCPA	Cucumber LHCP mRNA encoding ...	109	8e-21	
gi 8954292 gb AF139467.2 AF139467	Vigna radiata LHCI type ...	107	3e-20	
gi 169050 gb J01253.1 PEACAB15	Pea major chlorophyll a/b-bi...	107	3e-20	
gi 30696432 ref NM 124807.2	Arabidopsis thaliana light-har...	105	1e-19	U
gi 30696433 ref NM 124808.2	Arabidopsis thaliana myosin he...	105	1e-19	U
gi 4741951 gb AF134126.1	Arabidopsis thaliana chromosome V...	105	1e-19	U
gi 4741943 gb AF134122.1	Arabidopsis thaliana Lhcb2 protei...	105	1e-19	U
gi 13605753 gb AF361858.1	Arabidopsis thaliana AT5g54270/M...	105	1e-19	U
gi 16323261 gb AY057735.1	Arabidopsis thaliana AT5g54270/M...	105	1e-19	U
gi 15450493 gb AY052348.1	Arabidopsis thaliana At2g05100/F...	105	1e-19	U
gi 13926338 gb AF372917.1 AF372917	Arabidopsis thaliana AT5...	105	1e-19	U
gi 18556 emb X16536.1 GMCAB5	G.max DNA for Cab5	105	1e-19	
gi 18547 emb X12980.1 GMCAB2	Soybean Cab2 gene for minor PS...	105	1e-19	
gi 5002209 gb AF143691.1 AF143691	Arabidopsis thaliana type...	105	1e-19	U
gi 556366 gb L36064.1 PRULHP	Prunus persica (clone pAB19) l...	105	1e-19	
gi 2828180 dbj AB010695.1	Arabidopsis thaliana genomic DNA...	105	1e-19	
gi 20668 emb X56538.1 PSCABIIC	P.sativum Cab-8 gene for pho...	103	5e-19	
gi 1769848 emb Z75663.1 AGCHLABBP	A.graveolens mRNA for chl...	103	5e-19	
gi 1657858 gb U73218.1 TAU73218	Triticum aestivum chlorophy...	103	5e-19	U

gi 693915 gb U21111.1 STU21111	Solanum tuberosum chlorophyl...	103	5e-19	
gi 506628 gb U01964.1 GMU01964	Glycine max cv. Dare photosy...	103	5e-19	
gi 169054 gb K02067.1 PEACAB80	Pea (P.sativum) gene AB80 en...	103	5e-19	
gi 15637136 gb AF295639.1	Beta vulgaris chlorophyll a/b bi...	101	2e-18	
gi 2196771 gb AF003128.1 AF003128	Mesembryanthemum crystall...	101	2e-18	
gi 693913 gb U20983.1 STU20983	Solanum tuberosum chlorophyl...	101	2e-18	
gi 20938 emb X61915.1 PTCABP	P.thunbergii cab gene	101	2e-18	
gi 6635334 gb AF162200.1 AF162200	Lactuca sativa light-harv...	100	8e-18	
gi 693923 gb U21115.1 STU21115	Solanum tuberosum chlorophyl...	100	8e-18	
gi 217943 dbj D14002.1 LAUCAB	Lettuce mRNA for light-harves...	100	8e-18	
gi 30678159 ref NM_126540.2	Arabidopsis thaliana light-har...	98	3e-17	U
gi 4741947 gb AF134124.1	Arabidopsis thaliana Lhcb2 protei...	98	3e-17	U
gi 25054900 gb BT001933.1	Arabidopsis thaliana clone C1053...	98	3e-17	U
gi 15450348 gb AY052275.1	Arabidopsis thaliana At2g05100/F...	98	3e-17	U

## Alignments

Get selected sequences

Select all

Deselect all

>gi|29123377|gb|AY219853.1| Nicotiana tabacum chlorophyll a/b binding protein mRNA,  $\alpha$   
 cds  
 Length = 946

Score = 1715 bits (865), Expect = 0.0

Identities = 895/910 (98%)

Strand = Plus / Plus

```

Query: 1  atatcaaaactcaacagaaaaggnnnnnnnnngaagcttctttatctcaccagttnnnnnnn 60
        |||
Sbjct: 1  atatcaaaactcaacagaaaaggaaaaaaagaagcttctttatctcaccagttaaaaaaa 60

Query: 61  tggccacttctgcaattcaacaatctgcatttggtggccagacagtggctaaatcacaaa 120
        |||
Sbjct: 61  tggccacttctgcaattcaacaatctgcatttggtggccagacagtggctaaatcacaaa 120

Query: 121 atgagctagttaggaaaattggcagctttggcgggtggccgtgctaccatgagacgtaccg 180
        |||
Sbjct: 121 atgagctagttaggaaaattggcagctttggcgggtggccgtgctaccatgagacgtaccg 180

Query: 181 ttaaaagtgtcctcaaagcatctggtatggagaagaccgcccataatatttgggcccac 240
        |||
Sbjct: 181 ttaaaagtgtcctcaaagcatctggtatggagaagaccgcccataatatttgggcccac 240

Query: 241 tctctgagcaaactccatcatacctcaccggtgagttcccaggtgactatggttgggaca 300
        |||
Sbjct: 241 tctctgagcaaactccatcatacctcaccggtgagttcccaggtgactatggttgggaca 300

Query: 301 ctgctggactctcagctgacccagagacattcgctagaaaccgtgaacttgaggtgatcc 360
        |||
Sbjct: 301 ctgctggactctcagctgacccagagacattcgctagaaaccgtgaacttgaggtgatcc 360

Query: 361 attgccgttggggccatgcttggtgctttgggttggtgtctttcccgaaatcctttcaaaaa 420
        |||
Sbjct: 361 attgccgttggggccatgcttggtgctttgggttggtgtctttcccgaaatcctttcaaaaa 420

Query: 421 atggtgttaaattcggtgaagctgtttggttcaaggcaggagcccaaatcttctcggaag 480
        |||

```



## Inorganic Carbon Acquisition Systems in Cyanobacteria

### Minireview

Teruo Ogawa<sup>1,\*</sup> & Aaron Kaplan<sup>2</sup>

<sup>1</sup>Bioscience Center, Nagoya University, Chikusa, Nagoya 464-8601, Japan; <sup>2</sup>Department of Plant Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel; Author for correspondence (e-mail: ogawater@agr.nagoya-u.ac.jp; fax: 81-52-789-5214)

Received ■■■; accepted in revised form 24 April 2003

**Key words:** CO<sub>2</sub>-uptake, CO<sub>2</sub>-concentrating mechanism, cyanobacteria, HCO<sub>3</sub><sup>−</sup> transport, NADPH dehydrogenase

### Abstract

This minireview focuses on the mechanism of inorganic carbon uptake in cyanobacteria and in particular the two CO<sub>2</sub> uptake systems and two bicarbonate transporters recently identified in *Synechocystis* PCC 6803, and their presence in other cyanobacterial strains.

### Introduction

Cyanobacteria possess a CO<sub>2</sub>-concentrating mechanism (CCM) that enables efficient CO<sub>2</sub> fixation despite the low affinity of their Rubisco for CO<sub>2</sub> (Kaplan, Badger and Berry 1980; Ogawa, 1993; Kaplan and Reinhold 1999; Badger and Spalding 2000). The cellular components involved in the operation of the CCM include those engaged in inorganic carbon (Ci) uptake and accumulation, and the carboxysomes where most of the Rubisco and carbonic anhydrase (CA) are confined (Fukuzawa et al. 1992; Price, Colman and Badger 1992). It is well established that CO<sub>2</sub> and HCO<sub>3</sub><sup>−</sup> are actively taken up by separate, independent systems (Volkita et al. 1984; Espie, Miller and Canvin 1989; Miller, Espie and Canvin 1990, 1991). Generation of CO<sub>2</sub> from the HCO<sub>3</sub><sup>−</sup> accumulated within the cells is not catalyzed in the cytoplasm, and the Ci species do not reach equilibrium there (Reinhold, Zviman and Kaplan 1986; Price and Badger 1989). The accumulated HCO<sub>3</sub><sup>−</sup> penetrates the carboxysomes where CA catalyzes the formation of CO<sub>2</sub> in close proximity to Rubisco. In addition to compensating for the relatively low affinity of Rubisco for CO<sub>2</sub>, elevation of CO<sub>2</sub> concentration at the carboxylating site activates the enzyme and depresses photorespiration. The massive transmembrane Ci fluxes involved in the

operation of the CCM could help dissipate excess light energy. High-CO<sub>2</sub>-requiring mutants served as the main tool to identify genes involved in the operation of the CCM and to clarify many of the physiological processes. The first mutants isolated were impaired in various aspects related to the functional organization of the carboxysomes, these will not be discussed here (but see Marcus et al. 1986; Price et al. 1993; Ogawa, 1993; Kaplan and Reinhold 1999; Badger and Spalding 2000).

Recently, Ci-acquisition systems consisting of two CO<sub>2</sub>-uptake mechanisms and two bicarbonate transporters were identified in *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803) and *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* 7942) and mutants impaired in these activities, or possessing only one Ci-acquisition system, became available (Omata et al. 1999; Ohkawa, Pakrasi, Ogawa 2000; Shibata et al. 2001, 2002a, 2002b; Maeda et al. 2002). Use of these mutants enabled detailed analysis of the physiological characteristics of each system. In this minireview we describe physiological characteristics and phylogenetic analysis of the four Ci acquisition systems identified in *Synechocystis* 6803. For a more comprehensive background, the reader is referred to earlier reviews (Miller, Espie and Can-

vin 1990; Ogawa, 1993; Raven 1997; Kaplan and Reinhold 1999; Badger and Spalding 2000).

### Mutants defective in CO<sub>2</sub> uptake

It is widely accepted that light-dependent uptake of CO<sub>2</sub> in cyanobacteria involves conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> inside the cells (Volokita et al. 1984). It is assumed that this conversion is mediated by a component that possesses a carbonic anhydrase (CA)-like activity (Volokita et al. 1984; Kaplan and Reinhold 1999). Isolation of the high-CO<sub>2</sub>-requiring mutants of *Synechocystis* 6803, RKa and RKb, defective in CO<sub>2</sub> uptake (Ogawa 1990) constituted the first breakthrough in the molecular analysis of CO<sub>2</sub>-uptake systems in cyanobacteria. These mutants bear point mutations in *ndhB* and *ndhL* (renamed from *ictA*) encoding subunit proteins of NAD(P)H dehydrogenase (NDH-1) (Ogawa 1991a, 1991b, 1992). A  $\Delta ndhB$  mutant of *Synechococcus* 7942 also showed a high CO<sub>2</sub>-requiring phenotype (Marco et al. 1993). The observation that NDH-1 complexes involved in the conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> (see below) are localized on the thylakoid membrane (Ohkawa et al. 2001) suggested that CO<sub>2</sub> enters the cells by diffusion followed by conversion to HCO<sub>3</sub><sup>-</sup> by a thylakoid-located mechanism. Mass-spectrometric measurements showed that the light-dependent <sup>18</sup>O exchange between CO<sub>2</sub> and water was strongly impaired in the RKa and RKb mutants (Ogawa 1990). Further, application of a water-channel blocker strongly inhibited CO<sub>2</sub> uptake in *Synechococcus* 7942 suggesting that CO<sub>2</sub> enters the cells by diffusion via water channels (Tchernov et al. 2001). Taken together, these data are consistent with the proposal that the maintenance of the concentration gradient driving the diffusive CO<sub>2</sub> influx depends on the conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> in the cytoplasm. Since CO<sub>2</sub> uptake shows characteristics of saturable kinetics, the V<sub>max</sub> is likely to be set by the maximal activity of the converting system.

### Two functionally distinct NDH-1 complexes

Analysis of the genomic sequence of *Synechocystis* 6803 revealed the presence of multiple copies of *ndhD* and *ndhF*, although most of the other *ndh* genes are present as a single copy (Cyanobase; <http://www.kazusa.or.jp/cyano/>). NdhD and NdhF are both members of a larger family and may be related to

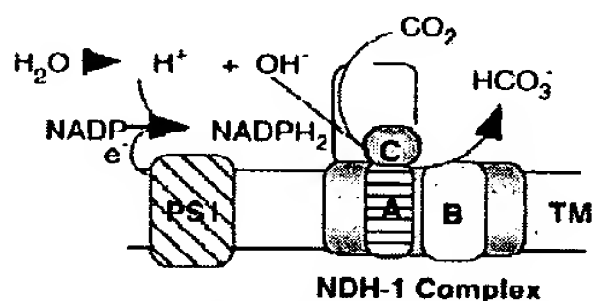
an ancient gene duplication event. Phylogenetic analysis indicated that at least four genes belong to the *ndhD* (*ndhD1*, *D2*, *D3* and *D4*) and three genes to the *ndhF* (*ndhF1*, *F3* and *F4*) families. Inactivation of each of the *ndhD* had little effect on the growth characteristics of the cells except that mutant  $\Delta ndhD3$  grew very slowly under extremely low CO<sub>2</sub> concentrations (i.e., 50  $\mu$ L/L CO<sub>2</sub>, Ohkawa et al. 1998; Price et al. 1998). Among the 4 *ndhD* genes, *ndhD1* and *ndhD3* are highly homologous to *ndhD2* and *ndhD4*, respectively. The double mutant,  $\Delta ndhD1/ndhD2$ , was unable to grow under photoheterotrophic conditions although it took up CO<sub>2</sub> in the light and grew normally under air levels of CO<sub>2</sub> (Ohkawa, Pakrasi and Ogawa 2000). In contrast, the double mutant  $\Delta ndhD3/ndhD4$  grew under photoheterotrophic conditions but was unable to take up CO<sub>2</sub> and to grow in air at pH 7.0 (Ohkawa, Pakrasi and Ogawa 2000). Thus, it became evident that *Synechocystis* 6803 possesses two functionally distinct NDH-1 complexes. The NDH-1 of the NdhD1/NdhD2 type mediates the electron transport from NADPH to plastoquinone and is an essential component of PS I-dependent cyclic electron flow. The contribution of NDH-1 type NdhD3/NdhD4 to this electron transport appears to be small. Table 1 summarizes the typical phenotypes of the  $\Delta ndhD1/ndhD2$  and  $\Delta ndhD3/ndhD4$  mutants. Notably, the  $\Delta ndhB$  mutant (M55) exhibited all the phenotypes shown by the two double mutants suggesting that, functionally, NdhB is located upstream of the NdhD1/NdhD2 and NdhD3/NdhD4 systems (Ogawa 1991a).

### Constitutive and low CO<sub>2</sub>-induced CO<sub>2</sub> uptake systems

Northern analysis of the expression of *ndhD* genes revealed that *ndhD2* and *ndhD3* are induced by low-CO<sub>2</sub> whereas *ndhD1* and *ndhD4* are constitutively expressed (Ohkawa et al. 1998). These data raised the possibility that two CO<sub>2</sub> uptake systems operate in *Synechocystis* 6803, one NdhD3-dependent induced under low-CO<sub>2</sub> conditions and a second constitutive, NdhD4-dependent system. RT-PCR analyses indicated that in *Synechocystis* 6803 *sll1732* (*ndhF3*), *sll1733* (*ndhD3*) and *sll1734* (*cupA*, CO<sub>2</sub> uptake A) are co-transcribed (Ohkawa et al. 2000). Mutants constructed by inactivation of each of these genes grew very slowly at 50 ppm CO<sub>2</sub> (Ohkawa et al. 1998) and mutants of *Synechococcus* PCC 7002 where *ndhD3*, *ndhF3*

Table 1. Phenotypes of mutants impaired in subunits of NADPH dehydrogenases

Phenotypes	M55 ( $\Delta$ ndhB)	$\Delta$ ndhD1/ ndhD2	$\Delta$ ndhD3/ ndhD4
Growth under photoheterotrophic conditions	no	no	yes
Respiration rate	low	low	high
Photosystem-1 cyclic electron transport	no	no	yes
Growth under low CO <sub>2</sub> at pH 7	no	yes	no
CO <sub>2</sub> uptake	no	yes	no



	Low CO <sub>2</sub> Induced	Constitutive
<b>Components</b>		
A	NdhD3	NdhD4
B	NdhF3	NdhF4
C	CupA	CupB
<b>K<sub>1/2</sub>(CO<sub>2</sub>)(<math>\mu</math>M)</b>		
H-cells	0.9	2.8
L-cells	0.9	2.8
<b>Vmax (<math>\mu</math>mol/mg Chl h)</b>		
H-cells	45	150
L-cells	320	135

Figure 1. A schematic model of the CO<sub>2</sub>-uptake systems, their kinetic parameters and the components involved. The kinetic parameters were calculated from CO<sub>2</sub> uptake experiments performed with whole cells.

or *cupA* were inactivated showed a similar phenotype (Price et al. 1998). Regardless of the CO<sub>2</sub> concentration during growth, these mutants exhibited the CO<sub>2</sub>-uptake characteristics of the constitutive system, i.e., relatively low affinity for CO<sub>2</sub> and low Vmax for CO<sub>2</sub> uptake (Klughammer et al. 1999; Ohkawa et al. 2000; Shibata et al. 2001, Figure 1). Transformation of a  $\Delta$ ndhD3 mutant with a transposon-bearing library enabled the isolation of mutants unable to grow in air at pH 7.0. These mutants bore the antibiotic-resistance tags within *slr1302*, designated *cupB* (a homologue of *cupA*, Shibata et al. 2001). Mutants constructed by inactivation of *slr0026* (*ndhF4*), *slr0027* (*ndhD4*) or *cupB* (but possessing normal NdhD3-NdhF3-CupA) showed CO<sub>2</sub>-uptake characteristics of the low-CO<sub>2</sub>-

induced system, i.e. higher affinity to CO<sub>2</sub> and Vmax typical of low CO<sub>2</sub>-grown cells (Figure 1). Double mutants impaired in one component of each of these systems, the high- (induced) and the low affinity (constitutive), demanded high CO<sub>2</sub> for growth (Shibata et al. 2001, 2002a). Figure 1 shows a schematic model of the CO<sub>2</sub>-uptake systems and the components involved. It also provides the kinetic parameters of the induced and the constitutive CO<sub>2</sub>-uptake systems calculated from uptake experiments performed with whole cells. As expected, the apparent affinity of the low-CO<sub>2</sub> induced system for CO<sub>2</sub> ( $K_{1/2}(\text{CO}_2) = 0.9 \mu\text{M}$ ) was significantly higher than that exhibited by the constitutive one ( $K_{1/2}(\text{CO}_2) = 2.8 \mu\text{M}$ ). Notably, while the curves relating CO<sub>2</sub> uptake to its concentration showed saturable kinetics, a significant extended linear phase was detected (Shibata et al. 2001) suggesting that CO<sub>2</sub> uptake was diffusion-limited and that the intrinsic  $k_m(\text{CO}_2)$  of the uptake systems is even lower. The very high affinity for CO<sub>2</sub> could explain the observation that low-CO<sub>2</sub>-grown cyanobacterial cells can utilize Ci from the medium almost to completion. It is also important to note (Figure 1) that the affinity of the low CO<sub>2</sub>-induced system for CO<sub>2</sub> did not change during acclimation of the cells after transfer from high to low-CO<sub>2</sub>. In contrast, the Vmax of uptake increased significantly during this acclimation. These data suggested that the acclimation to low-CO<sub>2</sub> involves a rise in the abundance of transporting entities rather than post-transcriptional modification of those already present under high CO<sub>2</sub> as earlier proposed for HCO<sub>3</sub><sup>-</sup> transport (Sültemeyer et al. 1998).

#### An ABC-type HCO<sub>3</sub><sup>-</sup> transporter (BCT1)

The finding of a low CO<sub>2</sub>-inducible 42 kD protein in the cytoplasmic membrane of *Synechococcus* 7942 was the first step towards molecular analysis of



Table 2. Growth characteristics of *Synechocystis* 6803 mutants impaired in various Ci acquisition system(s)

Mutants	In air at pH 7.0	In air at pH 9.0
$\Delta A$	+ <sup>a</sup>	+
$\Delta B$	+	+
$\Delta A/B$	-	+
$\Delta A/B/C$	-	+
$\Delta A/B/D$	-	-
$\Delta A/B/C/D$	-	-
$\Delta A/D$	+ <sup>a</sup>	+
$\Delta B/D$	+	+
$\Delta C/D$	+	+

A: Low CO<sub>2</sub>-induced CO<sub>2</sub> uptake system; B: Constitutive CO<sub>2</sub> uptake system; C: ABC-type HCO<sub>3</sub><sup>-</sup> transporter (BCT1); D: SbtA-type HCO<sub>3</sub><sup>-</sup> transporter.

<sup>a</sup>Growth was very slow at 20 ppm CO<sub>2</sub>.

HCO<sub>3</sub><sup>-</sup> transport (Omata and Ogawa 1985, 1986). A gene encoding this protein (*cmpA*, cytoplasmic membrane protein) was isolated and shown to form an operon with *cmpB*, *cmpC* and *cmpD* (Omata et al. 1990; Omata 1992). Sequence data indicated that these genes encoded subunits of an ABC-type transporter, *cmpA* being a substrate-binding subunit. Experimental evidence that the *cmp* operon encodes an ABC-type HCO<sub>3</sub><sup>-</sup> transporter was obtained after it was constitutively expressed in high CO<sub>2</sub>-grown cells (Omata et al. 1999). The *cmpA* protein obtained by expressing the gene in *E. coli* possessed HCO<sub>3</sub><sup>-</sup> binding capability (Maeda et al. 2000). The K<sub>1/2</sub> (HCO<sub>3</sub><sup>-</sup>) value for the BCT1-dependent HCO<sub>3</sub><sup>-</sup> transporter and the affinity of *cmpA* for HCO<sub>3</sub><sup>-</sup>, estimated by a mass spectrometry, were 15 and 5  $\mu$ M, respectively. Interestingly, inactivation of *cmp* genes in *Synechocystis* 6803, including strain  $\Delta ndhD3/\Delta ndhD4$ , hardly affected the HCO<sub>3</sub><sup>-</sup> transport activity and the mutant showed the growth characteristics of the wild types at pH 9.0. (Shibata et al. 2001). These data suggested that the *cmpA-D* HCO<sub>3</sub><sup>-</sup> transporter plays a minor role in *Synechocystis* 6803 and that another HCO<sub>3</sub><sup>-</sup> transporting system operates in this organism.

#### A sodium-dependent HCO<sub>3</sub><sup>-</sup> transporter

Cyanobacteria readily use CO<sub>2</sub> as a carbon source even at alkaline pH values, owing to their high affinity for CO<sub>2</sub> (particularly when grown under low CO<sub>2</sub> concentration). Therefore it has been difficult to detect and identify the HCO<sub>3</sub><sup>-</sup> transporting entities. The use

of mutants unable to take up CO<sub>2</sub> has proven helpful in this respect. The double mutant of *Synechocystis* 6803,  $\Delta ndhD3/\Delta ndhD4$ , was unable to take up CO<sub>2</sub> and hence to grow at pH 7.0 in air. On the other hand, at pH 9.0 it exhibited normal HCO<sub>3</sub><sup>-</sup>-transport activity and could grow like the wild type in air (Ohkawa et al. 2000), conditions where Ci is mainly supplied by HCO<sub>3</sub><sup>-</sup> transport. Sodium ions are essential for HCO<sub>3</sub><sup>-</sup> uptake by cyanobacteria (Reinhold, Zviman and Kaplan 1986; Espie and Kandasamy 1994; So et al. 1998) and hence for their growth (Miller, Turpin and Calvin 1984) particularly at alkaline pH values. Recently, it was shown that *slr1512*, designated *sbtA* (sodium-bicarbonate transporter), encodes a novel transporter involved in Na<sup>+</sup>-dependent HCO<sub>3</sub><sup>-</sup> uptake (Shibata et al. 2002b). Inactivation of *sbtA* in the wild type had no effect on the growth characteristics of cells grown under air in normal BG-11 medium. It is likely that under these conditions CO<sub>2</sub> uptake provided enough carbon to support growth. On the other hand, inactivation of *sbtA* in a  $\Delta ndhD3/\Delta ndhD4$  mutant (impaired in CO<sub>2</sub> acquisition) abolished Ci uptake almost completely and the cells were unable to grow in air. Disruption of *sbtA* in the single  $\Delta ndhD3$  or  $\Delta ndhD4$  mutants, which are able to take up CO<sub>2</sub> either by the constitutive or by the inducible systems, respectively (Shibata et al. 2001; Ohkawa et al. 2000a, b), hardly affected their growth. Table 2 shows that the presence of either low CO<sub>2</sub>-inducible or constitutive CO<sub>2</sub>-uptake systems is essential for growth at pH 7.0 in air. Most pronounced is the role of the low CO<sub>2</sub>-inducible system for growth under very low CO<sub>2</sub> concentrations, lower than 1.5  $\mu$ M CO<sub>2</sub>. On the other hand, the SbtA-type HCO<sub>3</sub><sup>-</sup> transporter was essential for growth at pH 9.0 in air when both CO<sub>2</sub>-uptake systems were inactivated. The contribution of the ABC-type HCO<sub>3</sub><sup>-</sup> transporter (BCT1) to *Synechocystis* 6803 growth is very small.

In high CO<sub>2</sub>-grown *Synechocystis* 6803, the abundance of the transcript originating from *sbtA* was very low and in most cases hardly detectable (Shibata et al. 2002b). The level of this transcript increased significantly within 2 to 6 h of exposure to air level of CO<sub>2</sub> concomitant with a large rise in HCO<sub>3</sub><sup>-</sup> transport activity. Maximal rate of SbtA-dependent HCO<sub>3</sub><sup>-</sup> uptake was reached at 100  $\mu$ M HCO<sub>3</sub><sup>-</sup> and the K<sub>1/2</sub>(HCO<sub>3</sub><sup>-</sup>) value was about 16  $\mu$ M (Figure 2). SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport was specifically dependent on the presence of Na<sup>+</sup> ions, maximal HCO<sub>3</sub><sup>-</sup> uptake was attained at 6 mM Na<sup>+</sup> and the concentration of Na<sup>+</sup> essential to support half maximal

$\text{HCO}_3^-$  transport was about 1 mM. Three different alternatives were proposed to explain the specific dependence of  $\text{HCO}_3^-$  transport on the presence of sodium ions in cyanobacteria (Kaplan et al. 1990; Espie and Kandasamy 1994). That  $\text{Na}^+$  is essential for the maintenance of the internal pH, via a  $\text{Na}^+/\text{H}^+$  antiporter, during  $\text{CO}_2$  fixation from imported  $\text{HCO}_3^-$ ; that  $\text{HCO}_3^-$  is transported via a  $\text{Na}^+/\text{HCO}_3^-$  symporter secondary to a primary  $\text{Na}^+$  pump that maintains the  $\Delta\mu\text{Na}^+$  essential for fueling  $\text{HCO}_3^-$  transport; and that  $\text{Na}^+$  binds to the  $\text{HCO}_3^-$  transporter and changes its affinity. The SbtA-dependent  $\text{HCO}_3^-$  uptake was strongly affected by the ambient pH, it was highest at pH 9 and the activity declined to about 50% and 20% at pH 8.0 and 7.0, respectively. This finding seems to exclude the possibility that the role of  $\text{Na}^+$  is in the maintenance of the internal pH. Inactivation of *slr1509* (*ntpJ*, encoding a protein homologous to a subunit of HKT1 in *Arabidopsis thaliana* that mediates  $\text{Na}^+$  transport, Uozumi et al. 2000) in the wild type abolished its ability to grow at  $\text{Na}^+$  concentration higher than 0.1 M; and that of mutant  $\Delta\text{ndhD3}/\text{ndhD4}$  to grow in air but not its ability to grow under 3%  $\text{CO}_2$ . The  $\text{HCO}_3^-$ -transport activity in the  $\Delta\text{ndhD3}/\text{ndhD4}/\text{ntpJ}$  mutant was only about one third that in the  $\Delta\text{ndhD3}/\text{ndhD4}$  mutant (Shibata et al. 2002b), supporting the notion that NtpJ is a subunit of a  $\text{Na}^+$ -extrusion pump essential for the SbtA-mediated  $\text{HCO}_3^-$  transport. The specific dependence of the SbtA-mediated  $\text{HCO}_3^-$  transport on  $[\text{Na}^+]$  and the impaired ability of  $\Delta\text{ntpJ}$  mutant to transport  $\text{HCO}_3^-$  point to  $\Delta\mu\text{Na}^+$  across the cytoplasmic membrane as the driving force for the SbtA-mediated  $\text{HCO}_3^-$  transport and suggest, therefore that SbtA functions as a  $\text{Na}^+/\text{HCO}_3^-$  transporter.

It was earlier proposed that IctB is involved in  $\text{HCO}_3^-$  uptake in *Synechococcus* 7942 suggesting that its homologue, Slr1515, may have a similar function in *Synechocystis* 6803 (Bonfil et al. 1998). However, lack of  $\text{HCO}_3^-$  uptake in the  $\Delta\text{ndhD3}/\text{ndhD4}/\text{sbtA}/\text{cmpA}$  mutant (Shibata et al. 2002b) did not lend support to this possibility. On the basis of results obtained by mass-spectrometry, it was concluded that *Synechococcus* sp. strain 7002 and *Synechococcus* 7942 possess a constitutive, low affinity,  $\text{HCO}_3^-$  transport system (Sültemeyer et al. 1998; Price et al. 2002). Interestingly, none of the  $\text{HCO}_3^-$  transporters recognized in *Synechocystis* 6803 (using mutants impaired in  $\text{CO}_2$  uptake) is constitutively expressed. Although we once observed low levels expression of *sbtA* in high  $\text{CO}_2$ -grown cells, repeated experiments indicated that the

transcript was hardly detectable in most cases. High  $\text{CO}_2$ -grown cells of *Synechocystis* 6803 did not exhibit low affinity  $\text{HCO}_3^-$  transport. This discrepancy might be due to differences between the various strains examined or the intrinsic difficulties involved in the assessment of  $\text{HCO}_3^-$  uptake by the mass spectrometry (see below).

#### Photosynthetic electron transport involved in $\text{CO}_2$ uptake and $\text{HCO}_3^-$ transport

Clearly, the active uptake and accumulation of Ci to values 1000-fold higher in the cells than in their medium, is driven by photosynthetic light energy. However, the specific role of the photosynthetic reaction centers and of various segments of the photosynthetic electron transport chain is not understood. Action spectra for Ci accumulation indicated that uptake can be energized by Photosystem I (PS I) alone. Mutants of *Synechocystis* 6803 (Ogawa 1991a, b; Mi et al. 1992, 1995) and *Synechococcus* 7942 (Marco et al. 1993) defective in *ndhB* and *ndhL* (encoding subunits of NAD(P)H dehydrogenase, NDH-1) do not exhibit either PS I-cyclic electron transport or  $\text{CO}_2$  uptake. Results obtained with these mutants supported the notion that NDH-1 dependent cyclic electron transport is essential to energize  $\text{CO}_2$  uptake (Ogawa 1993). However, the finding that cyanobacteria possess two functionally distinct NDH-1 complexes and that inactivation of NdhD1/NdhD2-type NDH-1 complex or NdhF(1)-type NDH-1 complex essential to cyclic electron transport had little effect on  $\text{CO}_2$  uptake (Sültemeyer et al. 1997; Ohkawa, Pakrasi and Ogawa 2000) made the direct involvement of cyclic PS I in  $\text{CO}_2$  uptake questionable.

Use of specific electron transport inhibitors and acceptors suggested that  $\text{CO}_2$  uptake in *Synechococcus* UTEX 625 is in fact supported by cyclic electron flow and that, in contrast,  $\text{HCO}_3^-$  transport depends on linear electron flow (Li and Calvin 1998). Draining of electrons from PS I to N, N-dimethyl-p-nitrosoaniline (PNDA) or methylviologen inhibited  $\text{CO}_2$  uptake but not  $\text{HCO}_3^-$  transport (Ogawa, Miyano and Inoue 1985; Li and Calvin 1998; Tchernov et al. 2001). In both *Synechocystis* 6803 and *Synechococcus* 7942,  $\text{CO}_2$  uptake by the low- $\text{CO}_2$ -inducible system was severely inhibited by DCMU but the constitutive system was less sensitive (Shibata et al. 2002a, Maeda et al. 2002). Inhibition of  $\text{CO}_2$  uptake by DCMU was alleviated by duroquinone (Tchernov



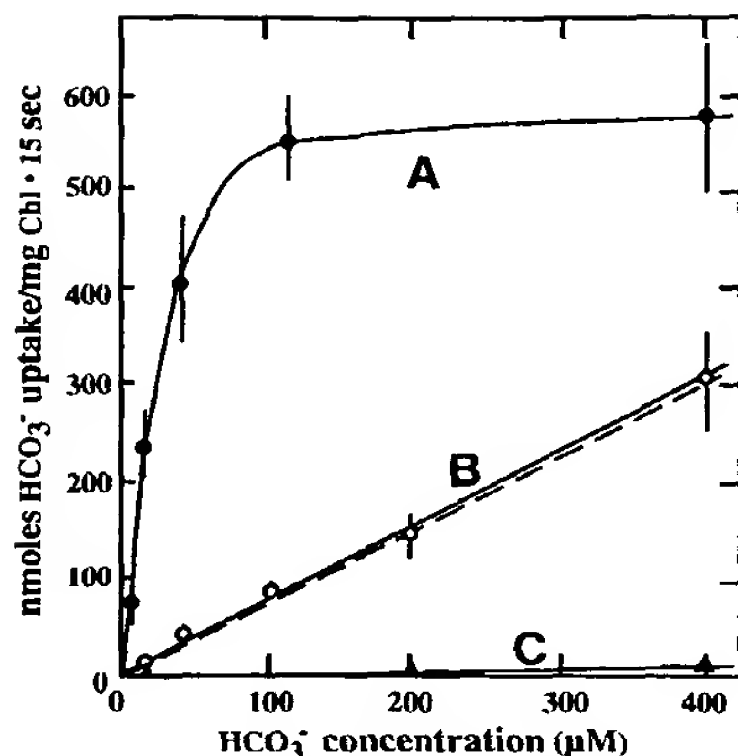


Figure 2. Uptake of  $\text{HCO}_3^-$  by the  $\Delta\text{ndhD3}/\text{ndhD4}/\text{cmpA}$  (A),  $\Delta\text{cmpA}/\text{sbtA}$  (B) and  $\Delta\text{ndhD3}/\text{ndhD4}/\text{cmpA}/\text{sbtA}$  (C) strains as affected by the  $\text{HCO}_3^-$  concentration. Uptake was measured in the medium containing 15 mM NaCl and various concentrations of  $\text{HCO}_3^-$  at pH 9.0. Vertical bars indicate standard deviations ( $n = 5$ ). Broken line shows the amount of  $\text{CO}_2$  that could be produced spontaneously from  $\text{HCO}_3^-$  at pH 9.0.

et al. 2001) and iodoacetamide, which blocked  $\text{CO}_2$  fixation and lowered the intracellular NADP/NADPH ratio (Ogawa, Miyano and Inoue 1985). It is possible that the low- $\text{CO}_2$ -inducible system is supported by PS I activity that depends on electrons donation from PS II (such as reduction of NADP) whereas the constitutive system is partially supported by cyclic PS I electron transport. The data at hand are all consistent with the notion that reduction of NADP to NADPH is essential for  $\text{CO}_2$  uptake, particularly that driven by the low- $\text{CO}_2$ -induced system. Since it has been proposed that conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  depends on local alkalization or removal of protons (Kaplan and Reinhold 1999; Maeda et al. 2002), ferredoxin-NADP reductase, thought to be involved in cyclic electron transfer under high energy demanding conditions (Matthijs et al. 2002), might play a role.

Light-driven net  $\text{CO}_2$  efflux during photosynthesis was observed in the marine *Synechococcus* strain WH 7803 consequent on dehydration of  $\text{HCO}_3^-$  within the cells (Tchernov et al. 1997).  $\text{CO}_2$  efflux was also observed in the light in *Synechococcus* strains UTEX 625 and PCC 7942 following inhibition of  $\text{CO}_2$  uptake by PNDA or methylviologen (Li and Calvin

1998; Tchernov et al. 2001). In contrast, in mutants capable of  $\text{HCO}_3^-$  transport but impaired in both the low  $\text{CO}_2$ -induced and the constitutive  $\text{CO}_2$ -uptake systems, efflux of  $\text{CO}_2$  due to dehydration of accumulated  $\text{HCO}_3^-$  within the cells was largely reduced. Taken together, the data suggested that the thylakoid-located conversion systems contribute importantly to the formation of  $\text{CO}_2$  from  $\text{HCO}_3^-$  within the cells (in addition to the carboxysomal-located CA) and that the direction of this CA-like activity (hydration or dehydration) is determined by the energization and redox states of the cells. The data also pointed to a massive Ci cycling between the cells and their medium driven by  $\text{CO}_2$ - and  $\text{HCO}_3^-$ -uptake systems (see Tchernov et al. this issue).

Both cyclic and linear electron transport lead to the formation of  $\Delta\mu\text{H}^+$  across the thylakoid membrane, essential for the formation of ATP. The latter can energize the ABC-type  $\text{HCO}_3^-$  transporter and may also be used to produce the  $\Delta\mu\text{Na}^+$  required to fuel the SbtA-dependent  $\text{HCO}_3^-$  transport. The mechanism by which  $\text{CO}_2$  uptake is energized is not clear but recent studies indicated that  $\Delta\mu\text{H}^+$  rather than ATP serves as the direct source of energy for the conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  (Tchernov et al. 2001). Conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  in alkaline domains could be used to energize  $\text{CO}_2$  uptake (Kaplan and Reinhold 1999). Formation of these domains is possibly coupled to reduction of NADP to NADPH on the thylakoid, or also envisaged as extraction of protons at CA sites on this membrane (Maeda et al. 2002).

#### Suitability of the methods used for measuring $\text{CO}_2$ and $\text{HCO}_3^-$ uptake

Isolation and characterization of mutants defective in  $\text{CO}_2$  or  $\text{HCO}_3^-$  uptake or both enable us to revisit some of the problems encountered in the application of the various methods used to measure these fluxes. The silicon-oil filtering centrifugation (Vlokhita et al. 1984) and the filtering method (Omata et al. 1999) were used to measure total Ci uptake. These methods can also be used to measure the initial rates of  $\text{CO}_2$  and of  $\text{HCO}_3^-$  uptake under isotopic disequilibrium conditions where labeled Ci is provided primarily as  $^{14}\text{CO}_2$  or  $\text{H}^{14}\text{CO}_3^-$  (Vlokhita et al. 1984; Miller, Espie and Calvin 1990). The filtering centrifugation method also enables the assessment of the kinetic parameters for the unidirectional influx of  $\text{CO}_2$  and of  $\text{HCO}_3^-$ . Use of the open gas-exchange method

(Ogawa, Miyano and Inoue 1985) allows accurate measurement of the concentration of  $\text{CO}_2$  leaving the cell suspension and hence calculation of the net rate of  $\text{CO}_2$  uptake since, at steady state, the concentration of  $\text{CO}_2$  in the medium remains constant. Use of this method showed that mutants capable of  $\text{HCO}_3^-$  uptake but unable to take up  $\text{CO}_2$  do not exhibit net  $\text{CO}_2$  uptake from the medium during photosynthesis (Ohkawa et al. 2000; Shibata et al. 2001). The mass-spectrometry method detects the change of  $\text{CO}_2$  concentration in the closed chamber with time of consumption or evolution (Badger, Palmqvist and Yu 1994). This method was also used to calculate the net contribution of  $\text{HCO}_3^-$  uptake to the photosynthetic activity. However, use of this approach to assess the actual amount of  $\text{HCO}_3^-$  transported and/or its kinetic parameters may lead to significant underestimations of the  $V_{\text{max}}$  and  $K_{1/2}(\text{HCO}_3^-)$  due to the fact that  $\text{HCO}_3^-$  efflux is substantial (Kaplan and Reinhold 1999; Tchernov et al. 1997; Tchernov et al. this issue) and not negligible as originally proposed.

Figure 2 shows the rate of  $\text{Ci}$  uptake by three types of *Synechocystis* 6803 mutants as affected by the  $\text{HCO}_3^-$  concentration, measured at pH 9.0 using the filtering method. There was no uptake of  $\text{Ci}$  in mutant  $\Delta\text{ndhD3}/\text{ndhD4}/\text{cmpA}/\text{sbtA}$  (where the  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake systems described above were inactivated) even at  $400 \mu\text{M}$   $\text{HCO}_3^-$ . These data suggested that *Synechocystis* 6803 does not possess an additional, possibly low affinity,  $\text{HCO}_3^-$  transporter. Mutant  $\Delta\text{ndhD3}/\text{ndhD4}/\text{cmpA}$  where only the SbtA-dependent  $\text{HCO}_3^-$  transport is functional showed saturable kinetics and reached maximum uptake activity at approximately  $100 \mu\text{M}$   $\text{HCO}_3^-$ . In contrast,  $\text{CO}_2$  uptake at pH 9.0 by mutant  $\Delta\text{cmpA}/\text{sbtA}$  (unable to take up  $\text{HCO}_3^-$  but possessing functional  $\text{CO}_2$ -uptake systems) increased linearly with the ambient  $\text{Ci}$  concentration (but note that the  $\text{CO}_2$  concentrations applied here were below the  $K_{1/2}$  for  $\text{CO}_2$  uptake; Shibata et al. 2001). The rate of  $\text{CO}_2$  uptake was similar to the maximal rate of uncatalyzed  $\text{CO}_2$  formation at this pH (Figure 2), indicating that uptake was rate-limited by the physicochemical conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  at the cell surface. In cyanobacteria, formation of  $\text{CO}_2$  from  $\text{HCO}_3^-$  in close vicinity to the cytoplasmic membrane might be catalyzed by a periplasmic-located carbonic anhydrase (Bedu, Beuf and Jose 1992) or accelerated by light-dependent proton extrusion that could acidify the periplasmic space (Kaplan, Lerner and Scherer 1989; Scherer, Hinrichs and Böger 1988; Katoh et al. 1996). If the cells take up

the  $\text{CO}_2$  as it is formed in the periplasmic space, the amount diffusing outwards to the bulk medium (where it can be detected by the mass spectrometer) will be greatly reduced. If this  $\text{CO}_2$  taken up is fixed in photosynthesis, it would erroneously be accounted for as  $\text{HCO}_3^-$  uptake.

### Phylogeny of the $\text{Ci}$ acquisition systems in cyanobacteria

The presence or absence of genes homologous to *ndhD3*, *ndhD4*, *ndhF3*, *ndhF4*, *cupA* and *cupB*, *sbtA* and *cmp* may provide better understanding of  $\text{Ci}$  uptake mechanisms in phytoplankton species and of their phylogenetic relationship. Inducible and constitutive  $\text{CO}_2$  uptake-systems appear to be present in a number of cyanobacteria. Involvement of NdhD3 and NdhF3 in high affinity  $\text{CO}_2$  uptake in *Synechococcus* sp. PCC 7002 has been reported (Klughammer et al. 1999). Mutants of *Synechococcus* 7942 impaired in *cupA* and *cupB* (in this organism designated *chpY* and *chpX*, respectively) also showed  $\text{CO}_2$  uptake characteristics of the constitutive and low  $\text{CO}_2$ -induced systems, respectively (Maeda et al. 2002; Price et al. 2002). The exact role of CupA and CupB is not known. In mutants of *Synechococcus* 7942 where both *cupA* and *cupB* were inactivated, inter-conversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  was slower than in the wild type. However, this does not necessarily indicate that the proteins encoded by these genes have a CA activity. In mutants RKa and RKb of *Synechocystis* 6803 likely to possess functional Cup proteins, inter-conversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  was strongly inhibited (Ogawa 1990). Moreover, we were unable to detect any CA activity with soluble CupA protein expressed in *E. coli* (unpublished). Thus, the naming of *chpX* and *chpY* ( $\text{CO}_2$  hydration protein), based on the mass-spectrometric measurement of the mutant cells, is not justified. The phylogenetic analysis of NdhD/NdhF suggested an evolutionary relationship between cyanobacterial *ndhD1/ndhD2*-type and *ndhD* genes in chloroplast genomes; also that the cyanobacterial *ndhF1* is related to the chloroplast *ndhF* (Shibata et al. 2001). The analysis indicated that the *ndhD3*- and *ndhD4*-types, the *ndhF3/ndhF4*-type and the *cupA/cupB*-type genes specifically engaged in  $\text{CO}_2$  uptake are present only in cyanobacteria. All the cyanobacterial species for which the whole genome sequence is available, with the exception of the marine *Synechococcus* and *Prochlorococcus marinus*, possess

Ci Acquisition Systems	<i>Synechocystis</i> PCC 6803	<i>Synechococcus</i> PCC 7942	<i>Synechococcus</i> PCC 7002	<i>Anabaena</i> PCC 7120	<i>Nostoc punctiforme</i>	<i>Thermosynechococcus elongatus</i>	<i>Gloeobacter violaceus</i>	Marine <i>Synechococcus</i> WH8502	<i>Prochlorococcus marinus</i> MED4	<i>Prochlorococcus marinus</i> MIT9313	Proteins involved
Low CO <sub>2</sub> induced CO <sub>2</sub> uptake system	Shaded	Shaded	Shaded	Shaded	Shaded	Shaded	Shaded	Open	Open	Open	NdhD3, NdhF3, CupA
Constitutive CO <sub>2</sub> uptake system	Shaded	Shaded	Shaded	Shaded	Shaded	Shaded	Shaded	Shaded	Open	Open	NdhD4, NdhF4, CupB
ABC-type HCO <sub>3</sub> <sup>-</sup> transporter (BCT1)	Shaded	Shaded	Open	Shaded	Shaded	Shaded	Shaded	Open	Open	Open	CmpA, B, C, D
SbtA-type HCO <sub>3</sub> <sup>-</sup> transporter	Checkered	Checkered	Checkered	Checkered	Open	Open	Open	Open	Open	Open	SbtA
Potential SbtA-type HCO <sub>3</sub> <sup>-</sup> transporter	Open	Open	Open	Checkered	Checkered	Open	Open	Open	Checkered	Checkered	SbtA homologue

Figure 3. Presence of Ci acquisition systems in various cyanobacterial strains. Shaded and open boxes indicate the presence and absence of each system, respectively.

both *ndhF3* and *ndhF4* genes and the *cupA*- and *cupB*-type genes (Figure 3). The marine *Synechococcus* does ~~not possess homologues of *ndhD3/ndhF3/cupA* genes~~ and, therefore, appears to be deprived of the low-CO<sub>2</sub>-inducible CO<sub>2</sub>-uptake system. The phylogenetic analysis also suggested that apart from passive uptake of CO<sub>2</sub> the low- and the high-light strains of *P. marinus*, MED4 and MIT9313, must rely on HCO<sub>3</sub><sup>-</sup> transport as the sole source of Ci since they lack the *ndh* genes involved in active CO<sub>2</sub> uptake typical of other cyanobacteria (and possess only the *ndhD1*-type essential for PS I cyclic electron transport).

The ABC-type HCO<sub>3</sub><sup>-</sup> transporter (BCT1) encoded by the *cmp* operon is present in *Synechococcus* 7942, *Synechocystis* 6803, *Anabaena* PCC 7120, *Nostoc punctiforme*, *Thermosynechococcus elongatus* and *Gloeobacter violaceus* but is absent in the marine cyanobacterial strains (*P. marinus* strains MED4 and

MIT9313 and *Synechococcus* sp. PCC 7002) (Omata et al. 2002). It is intriguing to speculate that marine organisms or halotolerants like *Synechocystis* 6803 that can rely on a primary Na<sup>+</sup> pump to establish a  $\Delta\mu\text{Na}^+$  as the driving force to fuel a Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> transporter whereas fresh water strains use ATP to fuel an ABC type pump to drive HCO<sub>3</sub><sup>-</sup> transport. Homologues of SbtA have been identified in *Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7002, *Anabaena* PCC 7120, *Nostoc punctiforme*, *P. marinus* strains MED4 and MIT9313 (Shibata et al. 2002b). There are two types of SbtA in cyanobacteria, one consisting of 370-374 and the other of 324-339 amino acids. *Anabaena* possesses both types of SbtA. The sequence homology between the 2 types of SbtA is relatively weak but analyses of hydrophobicity profiles indicated that both types contains 10 membrane-spanning domains that are structurally highly conserved. Whether the short

type SbtA is functioning as a  $\text{HCO}_3^-$  transporter is yet to be examined. As indicated, a SbtA-like  $\text{HCO}_3^-$  transporter was found in *P. marinus* strains; this important organism does not appear to possess any other Ci acquisition systems. Eukaryotic algae including *Chlamydomonas reinhardtii* possess light-dependent Ci uptake machinery (Kaplan and Reinhold, 1999; Badger and Spalding 2000). However, they probably depend on  $\text{CO}_2$ -uptake systems different from those functioning in cyanobacteria since homologues of the cyanobacterial *cup* genes were not detected there.

### Concluding remarks

The cyanobacterial CCM comprises processes which accumulate Ci within the cells and raise the concentration of  $\text{CO}_2$  at carboxylation sites within the carboxysomes. The past five years has seen significant advances in the recognition at molecular level of the systems engaged in  $\text{HCO}_3^-$  transport and  $\text{CO}_2$  uptake and in our understanding of their dependence on light energy. On the other hand, little progress has been made towards better understanding of the assemblage of the carboxysomes, although the first, and for many years the only, cyanobacterial high- $\text{CO}_2$ -requiring mutants were impaired in the structural organization of these bodies. Further, information is still missing on the nature of the  $\text{CO}_2$  sensor and the processes involved in the acclimation of cyanobacteria to changing ambient  $\text{CO}_2$  concentration. Recent identification of a transcription factor which plays a key role in the signal transduction pathway involved in the acclimation of *Chlamydomonas reinhardtii* to low  $\text{CO}_2$  (Xiang, Zhang and Weeks 2001; Fukuzawa et al. 2001) may open the way to identify similar proteins in cyanobacteria.

### Acknowledgements

We thank Dr Leonora Reinhold for many helpful discussions. Studies in our laboratories were supported by a Grant-in-Aid for Scientific Research (B) (2)(12440228), a grant for 'Research for the Future' Program (JSPS-RFTF97R16001) and a Grant-in-Aid for Scientific Research on Priority Areas (C) 'Genome Biology' to T.O. and grants from USA-Israel Binational Science Foundation (BSF), the German Ministerium für Bildung, Wissenschaft, Forschung,

und Technologie (BMFT) and the Israeli Ministry of Science and Technology (MOST) to A.K.

### References

- Badger MR, Palmqvist K and Yu JW (1994) Measurement of  $\text{CO}_2$  and  $\text{HCO}_3^-$  fluxes in cyanobacteria and microalgae during steady-state photosynthesis. *Physiol Plant* 90: 529–36
- Badger MR and Spalding MH (2000)  $\text{CO}_2$  acquisition, concentration and fixation in cyanobacteria and algae. In: Leegood RC, Sharkey TD and von Caemmerer S (eds) *Advances in Photosynthesis*, vol. 9. *Photosynthesis: Physiology and Metabolism*, pp 399–434. Kluwer Academic Publishers, Dordrecht, The Netherlands
- Bedu S, Beuf L and Jose F (1992) Membrane and soluble carbonic anhydrase activities in a cyanobacterium, *Synechocystis* PCC 6803. In: Murata N (ed) *Research in Photosynthesis*, pp 819–822. Kluwer Academic Publishers, Dordrecht, The Netherlands
- Bonfil DJ, Ronen-Tarazi M, Sültemeyer D, Lieman-Hurwitz J, Schatz D and Kaplan A (1998) A putative  $\text{HCO}_3^-$  transporter in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *FEBS Lett* 430: 236–240
- Espie GS and Kandasamy RA (1994) Monensin inhibition of  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport distinguishes it from  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  transport and provides evidence for  $\text{Na}^+/\text{HCO}_3^-$  symport in the cyanobacterium *Synechococcus* UTEX 625. *Plant Physiol* 104: 1419–1428
- Espie GS, Miller AG and Calvin DT (1989) Selective and reversible inhibition of active  $\text{CO}_2$  transport by hydrogen sulfide in a cyanobacterium. *Plant Physiol* 91: 389–394
- Fukuzawa H, Suzuki E, Komukai Y and Miyachi S (1992) A gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC 7942. *Proc Natl Acad Sci USA* 89: 4437–4441
- Fukuzawa H, Miura K, Ishizaki K, Kucho KI, Saito T, Kohinata T, Ohyama K (2001) *ccmI*, a regulatory gene controlling the induction of a carbon concentrating mechanism in *Chlamydomonas reinhardtii*, by sensing  $\text{CO}_2$  availability. *Proc Natl Acad Sci USA* 98: 5347–5352
- Kaplan A and Reinhold L (1999)  $\text{CO}_2$ -concentrating mechanisms in photosynthetic microorganisms. *Ann Rev Plant Physiol, Plant Mol Biol* 50: 539–570
- Kaplan A, Badger MR and Berry JA (1980) Photosynthesis and intracellular inorganic carbon pool in the blue-green algae *Anabaena variabilis*: Response to external  $\text{CO}_2$  concentration. *Planta* 149: 219–226
- Kaplan A, Lerner M and Scherer S (1989) Nature of the light-induced  $\text{H}^+$  efflux and  $\text{Na}^+$  uptake in cyanobacteria. *Plant Physiol* 89: 1220–1225
- Kaplan A, Schwarz R, Ariel R and Reinhold L (1990) The  $\text{CO}_2$  concentrating system in cyanobacteria: perspectives and prospects. In: Kanai R, Katoh S and Miyachi S (eds) *Regulation of Photosynthetic Processes*. *Bot Mag Tokyo Special Issue* 2: 53–72
- Katoh A, Sonoda M, Katoh H and Ogawa T (1996) Absence of light-induced proton efflux in a *cotA*-less mutant of *Synechocystis* sp. strain PCC6803. *J Bacteriol* 178: 5452–5455
- Klughammer B, Sültemeyer D, Badger MR and Price GD (1999) The involvement of NAD(P)H dehydrogenase subunits, NdhD3 and NdhF3, in high-affinity  $\text{CO}_2$  uptake in *Synechococcus* sp. PCC 7002 gives evidence for multiple NDH-I complexes with specific roles in cyanobacteria. *Mol Microbiol* 32: 1316–1332.

- Li Q and Calvin DT (1998) Energy sources for  $\text{HCO}_3^-$  and  $\text{CO}_2$  transport in air-grown cells of *Synechococcus* UTEX 625. *Plant Physiol* 116: 1125–32
- Maeda S, Price GD, Badger MR, Enomoto C and Omata T (2000) Bicarbonate binding activity of the *cmpA* protein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in active transport of bicarbonate. *J Biol Chem* 275: 20551–20555
- Maeda S, Badger MR and Price GD (2002) Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic  $\text{CO}_2$  hydration in the cyanobacterium, *Synechococcus* sp. PCC7942. *Mol Microbiol* 43: 425–435
- Marcus Y, Schwarz R, Friedberg D and Kaplan A (1986) High  $\text{CO}_2$  requiring mutant of *Anacystis nidulans* R2. *Plant Physiol* 82: 610–612
- Marco E, Ohad N, Schwarz R, Lieman-Hurwitz J, Gabay C and Kaplan A (1993) High  $\text{CO}_2$  concentration alleviates the block in photosynthetic electron transport in an *ndhB*-inactivated mutant of *Synechococcus* sp. PCC 7942. *Plant Physiol* 101: 1047–1053
- Matthijs HCP, Jeanjean R, Yenenenko N, Huisman J, Joset F and Hellingwerf KJ (2002) Hypothesis: Versatile function of ferredoxin-NADP<sup>+</sup> reductase in cyanobacteria provides regulation for transient Photosystem I-driven cyclic electron flow. *Funct Plant Biol* 29: 201–210
- Mi H, Endo T, Schreiber U, Ogawa T and Asada K (1992) Electron donation from cyclic and respiratory flows to the photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803. *Plant Cell Physiol* 33: 1233–1237
- Mi H, Endo T, Ogawa T and Asada K (1995) Thylakoid membrane-bound, NADPH-specific pyridine nucleotide dehydrogenase complex mediated cyclic electron transport in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* 36: 661–668
- Miller AG, Turpin DH and Calvin DT (1984)  $\text{Na}^+$  requirement for growth, photosynthesis and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis*. *J Bacteriol* 159: 100–106.
- Miller AG, Espie GS and Calvin DT (1990) Physiological aspects of  $\text{CO}_2$  and  $\text{HCO}_3^-$  transport by cyanobacteria: a review. *Can J Bot* 68: 1291–302
- Miller AG, Espie GE and Calvin DT (1991) The use of COS, a structural analog of  $\text{CO}_2$ , to study  $\text{CO}_2$  transport in the cyanobacterium *Synechococcus* UTEX 625. *Plant Physiol* 90: 1221–1231
- Ogawa T (1990) Mutants of *Synechocystis* PCC 6803 defective in inorganic carbon transport. *Plant Physiol* 94: 760–765
- Ogawa T (1991a) A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC 6803. *Proc Natl Acad Sci USA* 88: 4275–4279
- Ogawa T (1991b) Cloning and inactivation of a gene essential to inorganic carbon transport of *Synechocystis* PCC 6803. *Plant Physiol* 96: 280–284
- Ogawa T (1992) Identification and characterization of the *ictA/ndhL* gene product essential to inorganic carbon transport of *Synechocystis* PCC 6803. *Plant Physiol* 99: 1604–1608
- Ogawa T (1993) Molecular analysis of the  $\text{CO}_2$  concentrating mechanism in cyanobacteria. In: Yamamoto HY and Smith C (eds) *Photosynthetic Responses to the Environment*, pp. 113–125. American Society of Plant Physiologists, Rockville, Maryland
- Ogawa T, Miyano A and Inoue Y (1985) Photosystem-I driven inorganic carbon transport in the cyanobacterium, *Anacystis nidulans*. *Biochim Biophys Acta* 808: 77–84
- Ohkawa H, Sonoda M, Katoh H and Ogawa T (1998) The use of mutants in the analysis of the CCM in cyanobacteria. *Can J Bot* 76: 1025–1034
- Ohkawa H, Pakrasi HB and Ogawa T (2000) Two types of functionally distinct NAD(P)H dehydrogenases in *Synechocystis* sp. strain PCC6803. *J Biol Chem* 275: 31630–31634
- Ohkawa H, Price GD, Badger MR and Ogawa T (2000) Mutation of *ndh* genes leads to inhibition of  $\text{CO}_2$  uptake rather than  $\text{HCO}_3^-$  uptake in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* 182: 2591–2596
- Ohkawa H, Sonoda M, Shibata M and Ogawa T (2001) Localization of NAD(P)H dehydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803. *J Bacteriol* 183: 4938–4939
- Omata T (1992) Characterization of the downstream region of *cmpA*: Identification of a gene cluster encoding a putative permease of the cyanobacterium *Synechococcus* PCC7942. In: Murata N (ed) *Research in Photosynthesis*, pp. 807–810. Kluwer Academic Publishers, Dordrecht, The Netherlands
- Omata T and Ogawa T (1985) Changes in the polypeptide composition of the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* during adaptation to low  $\text{CO}_2$  conditions. *Plant Cell Physiol* 26: 1075–1081
- Omata T and Ogawa T (1986) Biosynthesis of a 42KD polypeptide in the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* strain R<sub>2</sub> during adaptation to low  $\text{CO}_2$  concentration. *Plant Physiol* 80: 525–30
- Omata T, Carlson TJ, Ogawa T and Piece J (1990) Sequencing and modification of the gene encoding the 42-kilodalton protein in the cytoplasmic membrane of *Synechococcus* PCC 7942. *Plant Physiol* 93: 305–311
- Omata T, Price GD, Badger MR, Okamura M, Gohta S and Ogawa T (1999) Identification of an ABC-Type bicarbonate transporter of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Proc Natl Acad Sci USA*, 96: 13571–13576
- Omata T, Takahashi Y, Yamaguchi O and Nishimura T (2002) Structure, function and regulation of the cyanobacterial high-affinity bicarbonate transporter, BCT1. *Funct Plant Biol* 29: 151–159
- Price GD and Badger MR (1989) Expression of human carbonic anhydrase in the cyanobacterium *Synechococcus* PCC 7942 creates a high carbon dioxide-requiring phenotype: evidence for a central role for carboxysomes in the carbon dioxide concentrating mechanism. *Plant Physiol* 91: 505–513
- Price GD, Coleman JR and Badger MR (1992) Association of carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium *Synechococcus* PCC 7942. *Plant Physiol* 100: 784–793
- Price GD, Howitt SM, Harrison K and Badger MR (1993) Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in carboxysome assembly and function. *J Bacteriol* 175:2871–2879
- Price GD, Klughammer B, Ludwig M and Badger MR (1998) The functioning of the  $\text{CO}_2$  concentrating mechanism in several cyanobacterial strains: A review of general physiological characteristics, genes, proteins and recent advances. *Can J Bot* 76: 973–1002
- Price GD, Maeda, S., Omata, T., and Badger, M.R. (2002) Modes of active inorganic carbon uptake in the cyanobacterium, *Synechococcus* sp. PCC 7942. *Funct Plant Biol* 29: 131–149.
- Raven JA (1997) Inorganic carbon acquisition by marine autotrophs. *Adv Bot Res* 27: 85–209
- Reinhold L, Zviman M and Kaplan A (1986) Inorganic carbon fluxes and photosynthesis in cyanobacteria—a quantitative model. In: Biggins J (ed) *Progress in Photosynthesis Research*, Vol 4, pp 289–296. Martinus Nijhoff Publishers, The Hague

- Scherer S, Riege H, and Böger P (1988) Light-induced proton release by the cyanobacterium *Anabaena variabilis* dependence on  $\text{CO}_2$  and  $\text{Na}^+$ . *Plant Physiol* 86: 939–941
- Shibata M, Ohkawa H, Kaneko T, Fukuzawa H, Tabata S, Kaplan A and Ogawa T (2001) Distinct constitutive and low  $\text{CO}_2$ -induced  $\text{CO}_2$  uptake systems in cyanobacteria: Genes involved and their phylogenetic relationship with homologous genes in other organisms. *Proc Natl Acad Sci USA* 98: 11789–11794
- Shibata M, Ohkawa H, Katoh H, Shimoyama M and Ogawa T (2002a) Two  $\text{CO}_2$  uptake systems in cyanobacteria: Four systems for inorganic carbon acquisition in *Synechocystis* sp. strain PCC 6803. *Funct Plant Biol* 29: 123–129.
- Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kaplan A and Ogawa T (2002b) Genes essential to sodium-dependent bicarbonate transport in cyanobacteria: Function and phylogenetic analysis. *J Biol Chem* 277: 18658–18664
- So AKC, Kassam A and Espie GS (1998)  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport in the cyanobacterium *Synechocystis* PCC 6803. *Can J Bot* 67: 1084–1091
- Sültemeyer D, Price GD, Bryant DA and Badger MR, (1997) *PsaE*- and *NdhF*-mediated electron transport affect bicarbonate transport rather than carbon dioxide uptake in the cyanobacterium *Synechococcus* sp. PCC 7002. *Planta* 201: 36–42
- Sültemeyer D, Klughammer B, Badger MR, Price GD (1998) Fast induction of high-affinity  $\text{HCO}_3^-$ -transport in cyanobacteria. *Plant Physiol* 116: 183–192
- Tchernov D, Hassidim M, Luz B, Sukenik A, Reinhold L and Kaplan, A. (1997) Sustained net  $\text{CO}_2$  evolution during photosynthesis by marine microorganisms. *Curr Biol* 7: 723–728
- Tchernov D, Helman Y, Keren N, Luz B, Ohad I, Reinhold L, Ogawa T and Kaplan A (2001) Passive entry of  $\text{CO}_2$  and its intracellular conversion to  $\text{HCO}_3^-$  in cyanobacteria are driven by a Photosystem I-generated  $\mu\text{H}^+$ . *J Biol Chem* 276: 23450–23455
- Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, Tsuboi A, Bakker EP, Nakamura T, and Schroeder JI (2000) The *Arabidopsis* *IsHKT1* gene homolog mediates inward  $\text{Na}^+$  currents in *xenopus laevis* oocytes and  $\text{Na}^+$  uptake in *Saccharomyces cerevisiae*. *Plant Physiol* 122: 1249–1259
- Volokita M, Zenvirth D, Kaplan A and Reinhold L (1984) Nature of the inorganic carbon species actively taken up by the cyanobacterium *Anabaena variabilis*. *Plant Physiol* 76: 599–602
- Xiang Y, Zhang J and Weeks DP (2001) The *cia5* gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii* *Proc Natl Acad Sci USA* 98: 5341–5346

**Genes Essential to Sodium-Dependent Bicarbonate Transport in Cyanobacteria:  
Function and Phylogenetic analysis\***

Mari Shibata<sup>‡</sup>, Hirokazu Katoh<sup>‡</sup>, Masatoshi Sonoda<sup>‡</sup>, Hiroshi Ohkawa<sup>‡</sup>, Masaya Shimoyama<sup>‡</sup>,  
Hideya Fukuzawa<sup>§</sup>, Aaron Kaplan<sup>¶</sup> and Teruo Ogawa<sup>‡, †</sup>

<sup>‡</sup>Bioscience Center, Nagoya University, Chikusa, Nagoya 464-8601, Japan, <sup>§</sup>Graduate School  
of Biostudies, Kyoto University, Sakyo, Kyoto 606-8502, Japan, <sup>¶</sup>Department of Plant  
Sciences, The Hebrew University, 91904 Jerusalem, Israel

**<sup>†</sup>Corresponding author:**

Professor Teruo Ogawa

Bioscience Center, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Tel: 81-52-789-5215; Fax: 81-52-789-5214; e-mail: [ogawater@agr.nagoya-u.ac.jp](mailto:ogawater@agr.nagoya-u.ac.jp)

Running title: Genes Essential to Sodium-Dependent Bicarbonate Transport



## SUMMARY

The cyanobacterium *Synechocystis* sp. strain PCC 6803 possesses two CO<sub>2</sub>-uptake systems and two HCO<sub>3</sub><sup>-</sup> transporters. We transformed a mutant impaired in CO<sub>2</sub> uptake and in *cmpA-D* encoding a HCO<sub>3</sub><sup>-</sup> transporter with a transposon-inactivation library, and recovered mutants unable to take up HCO<sub>3</sub><sup>-</sup> and grow in low CO<sub>2</sub> at pH 9.0. They are all tagged within *slr1512* (designated *sbtA*). We show that SbtA-mediated transport is induced by low CO<sub>2</sub>, requires Na<sup>+</sup>, and plays the major role in HCO<sub>3</sub><sup>-</sup> uptake in *Synechocystis*. Inactivation of *slr1509* (homologous to *ntpJ* encoding a Na<sup>+</sup>/K<sup>+</sup> translocating protein) abolished the ability of cells to grow at [Na<sup>+</sup>] higher than 100 mM and severely depressed the activity of the SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport. We propose that the SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport is driven by  $\Delta\mu\text{Na}^+$  across the plasma membrane, which is disrupted by inactivating *ntpJ*. Phylogenetic analyses indicated that two types of *sbtA* exist in various cyanobacterial strains, all of which possess *ntpJ*. The *sbtA* gene is the first one identified as essential to Na<sup>+</sup>-dependent HCO<sub>3</sub><sup>-</sup> transport in photosynthetic organisms and may play a crucial role in carbon acquisition when CO<sub>2</sub> supply is limited, or in *Prochlorococcus* strains that do not possess CO<sub>2</sub>-uptake systems or Cmp-dependent HCO<sub>3</sub><sup>-</sup> transport.

## INTRODUCTION

Growth of many photosynthetic microorganisms depends on the activity of a CO<sub>2</sub>-concentrating mechanism (CCM)<sup>1</sup> which raises the [CO<sub>2</sub>] in close proximity to ribulose-1,5-bisphosphate carboxylase/oxygenase and thereby enables efficient CO<sub>2</sub> fixation despite the low affinity of the enzyme for CO<sub>2</sub> (1, 2). In the cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803), the CCM involves active CO<sub>2</sub> uptake and HCO<sub>3</sub><sup>-</sup> transport. We have recently identified two systems for CO<sub>2</sub> uptake in *Synechocystis* 6803, one constitutive and the other inducible by low CO<sub>2</sub> (3). As deduced from phylogenetic analysis of proteins encoded by the genes involved, these CO<sub>2</sub>-uptake systems are present in various cyanobacteria with the exception of *Prochlorococcus marinus* (3). The inducible system that depends on NdhD3/NdhF3/CupA shows higher maximal activity and higher affinity for CO<sub>2</sub> than the constitutive, NdhD4/NdhF4/CupB-dependent system. Inactivation of two different genes, one encoding a component of the constitutive system, and the other a constituent of the inducible system, abolished CO<sub>2</sub>-uptake activity. The double mutants were unable to grow at pH 7.0 under air level of CO<sub>2</sub> (3, 4). In contrast, since the mutants possessed HCO<sub>3</sub><sup>-</sup> transport capability they could grow like the wild-type (WT) at pH 9.0 in air.

An ABC-type HCO<sub>3</sub><sup>-</sup> transporter encoded by *cmpABCD* has been identified in *Synechococcus* sp. strain PCC 7942 (thereafter *Synechococcus* 7942) (5). Inactivation of *cmp* genes in *Synechocystis* 6803, however, had little effect on the HCO<sub>3</sub><sup>-</sup> transport activity. This indicated that another HCO<sub>3</sub><sup>-</sup> transporter, yet unidentified, plays a central role in HCO<sub>3</sub><sup>-</sup> uptake. Sodium ions are essential for cyanobacterial growth, particularly at alkaline pH values (6), and they were implicated in HCO<sub>3</sub><sup>-</sup> uptake (7). These results are consistent with the suggestion that

a Na<sup>+</sup>-dependent HCO<sub>3</sub><sup>-</sup> transporter might be functioning in cyanobacteria (7-10). In this paper we bring evidence that *slr1512* (designated *sbtA* for sodium-bicarbonate transport A) and *slr1509* (*ntpJ*) are essential for Na<sup>+</sup>-dependent HCO<sub>3</sub><sup>-</sup> transport and that *sbtA* most likely encodes a novel HCO<sub>3</sub><sup>-</sup> transporter, the first one identified in photosynthetic organisms. We suggest that SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport could be driven by the electrochemical gradient of Na<sup>+</sup> across the plasma membrane, established by NtpJ.

## EXPERIMENTAL PROCEDURES

**Growth Conditions**—WT and mutant cells of *Synechocystis* 6803 were grown at 30°C in BG11 medium (11) containing 20 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES)-KOH, pH 9.0, and bubbled with either 3% CO<sub>2</sub> in air (v/v) or air alone. Solid medium contained BG11 buffered at pH 9.0 and was supplemented with 1.5% agar and 5 mM sodium thiosulfate. Continuous illumination was provided by fluorescent lamps (50 μmol photons m<sup>-2</sup> s<sup>-1</sup>; 400-700 nm).

**Construction and Isolation of Mutants**—B1 is the mutant where several nucleotides within *ndhB* were replaced, as previously described (12,13). This mutant does not take up CO<sub>2</sub> but showed normal HCO<sub>3</sub><sup>-</sup>-transport activity. Construction of mutants *ΔndhD3*, *ΔndhD4*, *ΔcmpA* and *ΔntpJ* has been described earlier (12) and/or deposited in the web site “CyanoMutants” (<http://www.kazusa.or.jp/cyano/mutants/>). Strains bearing multiple mutations were obtained following transformation of given *Synechocystis* 6803 mutants with the constructs used to generate other single mutants.

A Genomic Priming System (New England Biolabs) was used to mobilize a transposon containing chloramphenicol-resistance ( $\text{Cm}^R$ ) gene for random insertion into the DNA of 110 different cosmids, which contained DNA fragments of *Synechocystis* 6803 previously used for genomic sequencing (14). The B1/ $\Delta\text{cmpA}$  mutant, defective in active  $\text{CO}_2$  uptake, was transformed with this transposon inactivation library. Colonies formed on plates containing chloramphenicol, kanamycin and spectinomycin were transferred to duplicate plates buffered at pH 9.0 containing the same drugs. One plate was placed under 3%  $\text{CO}_2$  in air (v/v) and the other in air alone. Mutants growing under 3%  $\text{CO}_2$ , but not in air, were recovered. The exact position of the  $\text{Cm}^R$  cassette in the mutant genome was determined as described previously (3).

*Measurements of  $\text{HCO}_3^-$  Uptake and  $\text{O}_2$  Evolution*—The rate of  $\text{HCO}_3^-$  uptake was measured using  $\text{H}^{14}\text{CO}_3^-$  in an assay buffer (50 mM CHES-KOH for pH 9.0 or N-Tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid-KOH for pH 7.0 and 8.0 containing 15 mM NaCl, 0.3 mM  $\text{MgSO}_4$ , 0.26 mM  $\text{CaCl}_2$  and 0.22 mM  $\text{K}_2\text{HPO}_4$ ) as previously reported (5).  $\text{HCO}_3^-$  uptake was initiated by the addition of  $\text{NaH}^{14}\text{CO}_3/\text{KHCO}_3$ . The sample was immediately illuminated with white light ( $400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). Uptake was terminated by rapid filtration of the cells onto a glass filter (GF/B, Whatman) by suction, followed by immediate washing of the filter with 5 ml of the assay buffer. Oxygen evolution was measured with an  $\text{O}_2$  electrode (Rank Brothers, Cambridge, England) on cells suspended in BG11 medium (pH 9.0) containing 15 mM NaCl. Cell suspensions (corresponding to Chl concentration of  $10 \mu\text{g/ml}$ ) were illuminated with white light ( $400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and, when  $\text{O}_2$  evolution ceased,  $\text{NaHCO}_3^-$  was added stepwise to attain the final concentrations of 5, 15, 30, 100 and  $400 \mu\text{M}$ ,

respectively.

*RT-PCR Analysis of Expression*—The amount of transcripts was evaluated by the RT-PCR method (15). RNAs were extracted from *Synechocystis* 6803 cells grown under 3 % CO<sub>2</sub> or after 2 and 6 hrs of bubbling with air, by the method of Aiba et al. (16), treated with RNase-free DNase I (Boehringer Mannheim) and then purified by phenol/chloroform extraction and ethanol precipitation. Reverse-transcription reaction was performed using superscript II (Gibco BRL) and reverse primers. The products were amplified by PCR and then analyzed by electrophoresis on 0.8 % agarose gel. Primers were designed so that the amplified products would be internal to the coding region of the genes. All the forward primers were designed for the sequences downstream of the translation initiation codon and the reverse primers to obtain the PCR products of about 350 bp. RNaseP gene was used as a control template (17). Reverse transcriptase was omitted from the RT reaction mixture to confirm the absence of contamination of genomic DNA.

*Other Methods*—Procedures previously described were used for the measurement of comparative cell growth on agar plates buffered at pH 9.0 (4, 12).

## RESULTS

*A Gene Involved in a Novel HCO<sub>3</sub><sup>-</sup> Transport System*—To isolate novel mutants impaired in HCO<sub>3</sub><sup>-</sup> uptake in *Synechocystis* 6803 and identify the relevant genes, it was essential to use strains defective in both CO<sub>2</sub> uptake and in the *cmp* operon that encodes an ABC-type HCO<sub>3</sub><sup>-</sup> transporter (5). The B1 strain, impaired in *ndhB*, was selected as a proper host since it is unable to take up CO<sub>2</sub> and does not grow at pH 7.0 under air level of CO<sub>2</sub> (12, 13). On the

other hand, this mutant exhibited normal  $\text{HCO}_3^-$ -transport activity and could grow like the WT in air at pH 9.0 (12), conditions where inorganic carbon (Ci) is mainly supplied by  $\text{HCO}_3^-$  transport. Inactivation of the *cmp* operon in the B1 mutant did not change its growth characteristics at pH 9.0, under either high or low levels of  $\text{CO}_2$  (not shown), suggesting that  $\text{HCO}_3^-$  uptake capability was not impaired. We transformed the double mutant B1/ $\Delta\text{cmpA}$  with a transposon-bearing inactivation library (3) and isolated four mutants defective in their ability to grow at pH 9.0 under air level of  $\text{CO}_2$  and unable to take up  $\text{HCO}_3^-$ . All these mutants (NB-3, 9, 10 and 48) had  $\text{Cm}^R$  cassettes at various sites within a single gene, *slr1512* (designated *sbtA*, Fig. 1A). WT *Synechocystis* 6803 and the  $\Delta\text{ndhD3}$ ,  $\Delta\text{ndhD4}$ ,  $\Delta\text{ndhD3/ndhD4}$  (hereafter  $\Delta\text{ndhD3/D4}$ ) and  $\Delta\text{ndhD3/D4/cmpA}$  mutants were transformed with the genomic DNA from strain NB-3 in order to interrupt their *sbtA*. As shown in Fig. 1B, all the mutants obtained, with the exception of  $\Delta\text{ndhD3/D4/sbtA}$  and  $\Delta\text{ndhD3/D4/cmpA/sbtA}$  (not shown), grew like the WT at pH 9.0 in air and in 3%  $\text{CO}_2$ . Inactivation of *sbtA* and/or *cmpA* in WT cells had no effect on their growth (not shown) presumably since the mutants were able to take up sufficient  $\text{CO}_2$  to support their growth. Similarly, disruption of *sbtA* in the single  $\Delta\text{ndhD3}$  or  $\Delta\text{ndhD4}$  mutants, which are able to take up  $\text{CO}_2$  either by the constitutive or by the inducible systems (3,4,12), had no effect on their growth (upper panel, Fig. 1B). It is most likely that the ability to take up  $\text{HCO}_3^-$  enabled growth of the  $\Delta\text{ndhD3/D4}$  mutant at alkaline pH and air level of  $\text{CO}_2$ . However, inactivation of *sbtA* in this double mutant resulted in the loss of its ability to grow under low  $\text{CO}_2$  even at pH 9 (Fig. 1B). These results suggested that the gene product of *sbtA* is involved in  $\text{HCO}_3^-$  transport and that its activity could support growth of the  $\Delta\text{ndhD3/D4}$  mutant, particularly at pH 9.0. In contrast to the  $\Delta\text{ndhD3/D4/sbtA}$

mutant, inactivation of *cmpA* in the  $\Delta ndhD3/D4$  strain scarcely affected its growth (Fig. 1B). These results indicated that the contribution of the Cmp-dependent  $\text{HCO}_3^-$  transport to the growth of *Synechocystis* 6803 is negligible. All mutants examined, with the exception of  $\Delta ndhD3/D4/sbtA$  (lower panel in Fig. 1B) and  $\Delta ndhD3/D4/sbtA/cmpA$  (not shown) grew like the WT on agar plates under 3%  $\text{CO}_2$ . The latter mutants could grow like the WT in liquid medium at pH 9.0 in 3%  $\text{CO}_2$  in air (v/v) but not in air alone (Fig. 1C).

Inactivation of the *slr1513* gene, located downstream of *sbtA* (Fig. 1A), within the  $\Delta ndhD3/D4$  mutant had no effect on growth performance (not shown). This result ruled out a possible pleiotrophic effect due to interruption of *sbtA*. The possibility that *sbtA* encodes a novel  $\text{HCO}_3^-$  transporter was examined further by measuring the activity of  $\text{HCO}_3^-$  transport and the expression of *sbtA* in the WT and the mutants (Figs. 2 and 3).

*A Low- $\text{CO}_2$  Inducible,  $\text{Na}^+$ -Dependent  $\text{HCO}_3^-$  Transport is Mediated by SbtA*—Figure 2A shows the amounts of  $\text{HCO}_3^-$  taken up by WT and various mutants during 15 sec incubation with 400  $\mu\text{M}$   $\text{HCO}_3^-$ . There was no significant difference between the amounts of  $\text{HCO}_3^-$  taken up by the WT and by mutants  $\Delta ndhD3/D4$ ,  $\Delta ndhD3/D4/cmpA$  (a, b and c).  $\text{HCO}_3^-$  uptake by  $\Delta ndhD3/D4/cmpA$  was about six times higher in light than in darkness (c and c'). Inactivation of *sbtA* in  $\Delta ndhD3/D4$  severely depressed the rate of  $\text{HCO}_3^-$  uptake (d and d'); disruption of *cmpA* in  $\Delta ndhD3/D4/sbtA$  reduced the  $\text{HCO}_3^-$ -transport activity somewhat further (e and e'). The low level of  $\text{HCO}_3^-$  uptake observed in  $\Delta ndhD3/D4/sbtA/cmpA$  most likely reflected non-specific adherence of  $^{14}\text{Ci}$  to the cells since light did not stimulate this apparent uptake. These data indicated that the SbtA-mediated system plays the major role in  $\text{HCO}_3^-$  uptake in *Synechocystis* 6803 and that the contribution of the Cmp-mediated  $\text{HCO}_3^-$  transport was very

small. This is in agreement with the ability of  $\Delta ndhD3/D4/cmpA$ , but not  $\Delta ndhD3/D4/sbtA$  and  $\Delta ndhD3/D4/cmpA/sbtA$ , to grow under low  $[CO_2]$  (Fig. 1B).

A small amount of transcript originating from *sbtA* was detected in the WT and  $\Delta ndhD3/D4$  mutant cells of *Synechocystis* 6803 grown under 3%  $CO_2$  (H-cells, lanes a and d for *sbtA* in Fig. 3) but the transcript abundance increased significantly within 2 to 6 hrs of exposure to air level of  $CO_2$  (lanes b, c, e and f for *sbtA*). This data indicated that expression of *sbtA* was induced by low  $CO_2$  in the WT and  $\Delta ndhD3/D4$  mutant, in agreement with the large rise in  $HCO_3^-$  transport activity in cells acclimated to air level of  $CO_2$  (c, f and i for L-cells versus h for H-cells in Fig. 2). A transcript of *cmpA* was not detectable in H-cells of the WT and  $\Delta ndhD3/D4$  mutant (Fig. 3, lanes a and d for *cmpA*) but was detected in the WT cells acclimated to air for 6 hrs (lane c for *cmpA*). The *cmpA* transcript was barely detectable in the mutant even after 6 hrs of acclimation to air (lane f for *cmpA*). This may explain the very low activity of the Cmp-dependent  $HCO_3^-$  transport in the  $\Delta ndhD3/D4/sbtA$  mutant (Fig. 2, d). The SbtA-dependent  $HCO_3^-$  uptake was strongly affected by the ambient pH level. At pHs 8.0 (Fig. 2, j) and 7.0 (k),  $HCO_3^-$  uptake was about 50 and 20 %, respectively, that observed at pH 9.0 (i). SbtA-mediated  $HCO_3^-$  transport was almost completely abolished when NaCl in the medium was replaced with KCl (g), indicating that  $HCO_3^-$  transport is specifically dependent on the presence of  $Na^+$  ions. Figures 4A and 4B show the dependency of the SbtA-mediated  $HCO_3^-$  transport in the  $\Delta ndhD3/D4/cmpA$  mutant to  $HCO_3^-$  and  $Na^+$  concentrations, respectively. Maximal rate of  $HCO_3^-$  uptake was reached at 100  $\mu M$   $HCO_3^-$  and the  $K_{1/2}(HCO_3^-)$  value was about 16  $\mu M$  (open circles in Fig. 4A). Photosynthetic  $O_2$  evolution displayed a similar dependency on external  $[HCO_3^-]$  (closed circles), suggesting that in this



mutant photosynthesis was rate-limited by the SbtA-mediated  $\text{HCO}_3^-$  transport. Dependency of the SbtA-mediated  $\text{HCO}_3^-$  transport on ambient  $[\text{Na}^+]$  was further supported by the nature of the curve relating  $\text{HCO}_3^-$  uptake to  $[\text{Na}^+]$  (Fig. 4B). Maximal  $\text{HCO}_3^-$  uptake was attained at 6 mM  $\text{Na}^+$  and the concentration of  $\text{Na}^+$  essential to support half maximal  $\text{HCO}_3^-$  transport was about 1 mM (Fig. 4B). These results are in general agreement with an earlier report (10) on the response of  $\text{HCO}_3^-$  uptake in *Synechocystis* 6803 to the presence of  $\text{Na}^+$ . The higher maximal rate of  $\text{HCO}_3^-$  uptake observed before was, most likely, due to simultaneous uptake of  $\text{CO}_2$  in WT where both the constitutive and the inducible  $\text{CO}_2$ -uptake systems (3) are functional. Furthermore, analysis of  $\text{CO}_2$  uptake by mutant  $\Delta\text{cmpA/sbtA}$  (unable to take up  $\text{HCO}_3^-$ , Fig. 4C) showed that it increased linearly with the ambient  $[\text{HCO}_3^-]$  well above the amount of  $\text{CO}_2$  that could be produced spontaneously from  $\text{HCO}_3^-$  at pH 9.0 (broken line). These data clearly indicated that conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  at the cell surface is faster than expected from physicochemical considerations based on the concentration of  $\text{HCO}_3^-$  and pH in the bulk medium. Formation of  $\text{CO}_2$  may be catalyzed by a periplasmic-located carbonic anhydrase (18) or accelerated by light-dependent proton extrusion that could acidify the periplasmic space (8, 19, 20).

*NtpJ is Involved in  $\text{HCO}_3^-$  Transport*—The specific dependence of the SbtA-mediated  $\text{HCO}_3^-$  transport on  $[\text{Na}^+]$  (Figs. 2B and 4B) recalls earlier studies (7, 9, 10, 21) where various possibilities were raised to explain the role of  $\text{Na}^+$ . If the  $\Delta\mu\text{Na}^+$  across the cytoplasmic membrane is essential for the operation of the SbtA-mediated  $\text{HCO}_3^-$  transport, inactivation of components involved in  $\text{Na}^+$  extrusion (primary  $\text{Na}^+$  or  $\text{Na}^+/\text{H}^+$  pumps) should affect the  $\text{HCO}_3^-$  uptake and growth of a mutant unable to utilize  $\text{CO}_2$  such as  $\Delta\text{ndhD3/D4}$  (Fig. 5).

*Synechocystis* 6803 can grow under a relatively high [NaCl] even exceeding 0.5 M (22). Inactivation of *slr1509* (*ntpJ*), encoding a protein that belongs to a Na<sup>+</sup>-transporter family (<http://motif.genome.ad.jp/>), barely affected the growth of *Synechocystis* 6803 in BG11 medium at pH 9.0 in air, but growth was severely depressed when [NaCl] was raised above 100 mM (Fig. 5A). These results suggested that NtpJ could be involved in Na<sup>+</sup> extrusion and that failure of the mutant to extrude Na<sup>+</sup> abolished its growth at elevated [NaCl]. In contrast to the WT, inactivation of *ntpJ* completely abolished growth of the  $\Delta ndhD3/D4$  mutant even in BG11 medium in air (Fig. 5B). On the other hand, under 3% CO<sub>2</sub>, the  $\Delta ndhD3/D4/ntpJ$  mutant grew almost like the WT (Fig. 5B). These results suggested involvement of NtpJ in the supply of Ci for growth. This was confirmed by measuring the uptake of HCO<sub>3</sub><sup>-</sup> uptake by this mutant (Fig. 5C). The HCO<sub>3</sub><sup>-</sup>-transport activity in the  $\Delta ndhD3/D4/ntpJ$  mutant was only about one third of that in the  $\Delta ndhD3/D4$  mutant (Fig. 5C) and became much lower during longer exposure of the mutant to light. These results support the notion that NtpJ is a subunit of a Na<sup>+</sup> extrusion pump essential for the SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport.

*Phylogenetic Analysis of SbtA and NtpJ in Cyanobacteria*—Homologues of SbtA have been identified in *Synechococcus* sp. PCC 6301 (Dr. M. Sugita, personal communication), *Synechococcus* sp. PCC 7002 (Drs. J. Zhao and D. Bryant, personal communication), *Anabaena* PCC 7120 (<http://www.kazusa.or.jp/cyano/>), *Nostoc punctiforme*, *P. marinus* strains MED4 and MIT9313 ([http://www.jgi.doe.gov/tempweb/JGI\\_microbial/html/index.html](http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html)) and in the non-photosynthetic bacteria *Mycobacterium tuberculosis* (23), *Caulobacter crescentus* (24) and *Bacillus halodurans* (25). The phylogenetic tree (Fig. 6A) pointed to two types of SbtA in cyanobacteria, one consisting of 370-374 and the other of 324-339 amino acids.

*Anabaena* possesses both types of SbtA. The sequence homology between the two types of SbtA is relatively weak but analyses of hydrophobicity profiles indicated that both types contains 10 membrane-spanning domains that are structurally highly conserved (Fig. 7). Search for specific motifs with the aid of TargetP program (26) identified a signal polypeptide sequence in the N-terminal region of both types of SbtA, likely to target them to the cell exterior and/or the thylakoid lumen. Presently, the exact location of the SbtA is not known, but based on the data presented here and its involvement in  $\text{Na}^+$  exchange, it is most likely located on the cytoplasmic membrane.

All the cyanobacterial strains investigated possess the NtpJ essential for the operation of the SbtA-mediated  $\text{HCO}_3^-$  transport. The phylogenetic tree of NtpJ indicated two types of proteins, one present in both strains of *P. marinus* and the other in the other organisms (Fig. 6B).

## DISCUSSION

*Four Systems for Ci Acquisition in Synechocystis 6803*—*Synechocystis* 6803 appears to possess four different systems for Ci acquisition. Two of them, recently identified, are engaged in  $\text{CO}_2$  uptake (3). The other two, involved in  $\text{HCO}_3^-$  transport, are the ABC-type transporter encoded by *cmpA-D* (5) and the SbtA-mediated system identified here. It was essential to inactivate both  $\text{CO}_2$ -uptake systems to recover the  $\Delta\text{sbtA}$  mutants since presence of either of them enabled photoautotrophic growth even at pH 9.0 in air (Fig. 1B). Measurements of growth and of  $\text{HCO}_3^-$  uptake (Figs. 1 and 2) indicated that SbtA plays the central role in  $\text{HCO}_3^-$  transport in *Synechocystis* 6803 and that the contribution of the CmpABCD-mediated  $\text{HCO}_3^-$

transport is negligible, also in mutant *AndhD3/D4*. Furthermore, lack of  $\text{HCO}_3^-$  uptake in the *AndhD3/D4/sbtA/cmpA* mutant ruled out the possibility that Slr1515 (homolog of IctB from *Synechococcus* 7942, ref. 27) is an independent  $\text{HCO}_3^-$  transporter in *Synechocystis* 6803. The role of Slr1515 (IctB) in intracellular  $\text{HCO}_3^-$  accumulation in cyanobacteria is not known and we were unable to inactivate *slr1515* in *Synechocystis* 6803. The inability to inactivate *ictB* (or its homologue, *slr1515*) suggests that its gene product plays a very important role. Based upon the observations presented here, one might expect that this protein act downstream from SbtA/CmpA/NdhD3/NdhD4. Enhancement of the expression of *sbtA* by low  $\text{CO}_2$  (Fig. 3) was in agreement with the considerable rise in  $\text{HCO}_3^-$  transport capability in cells grown under these conditions (Fig. 2).

*The Nature and Mode of Energization of the SbtA-Mediated  $\text{HCO}_3^-$  Transport*—Data presented here may help to identify the primary pump involved in the SbtA-mediated active  $\text{HCO}_3^-$  transport. SbtA does not possess an ATP-binding domain. It is therefore unlikely that ATP directly fuels it. SbtA-mediated  $\text{HCO}_3^-$  transport was strongly and specifically dependent on the presence of  $\text{Na}^+$  ions (Figs. 2B and 4B) and NtpJ was essential for both the growth of *Synechocystis* 6803 in the presence of elevated  $[\text{Na}^+]$  and for  $\text{HCO}_3^-$  transport (Fig. 5). These data are consistent with the suggestion that SbtA is a component of a  $\text{Na}^+/\text{HCO}_3^-$  symporter that drives the  $\text{HCO}_3^-$  transport secondary to a primary  $\text{Na}^+$  pump (7, 9, 10). The latter is essential to establish the  $\Delta\mu\text{Na}^+$  for active  $\text{HCO}_3^-$  accumulation. The nature of this primary sodium extrusion pump (28) is not known but NtpJ is likely to be involved. Measurements of the  $\Delta\mu\text{Na}^+$  value and of the  $\text{Na}^+$  flux across the cytoplasmic membrane of *Synechocystis* 6803, as affected by  $[\text{Na}^+]$ ,  $[\text{HCO}_3^-]$  and pH, are not available. In a detailed study, Ritchie et al (21)

measured some of these parameters in *Synechococcus* 7942. They concluded that  $\Delta\mu\text{Na}^+$  would be large enough to drive  $\text{HCO}_3^-$  uptake if the stoichiometry of  $\text{Na}^+:\text{HCO}_3^-$  is 2 or 3:1. Since the internal  $\text{HCO}_3^-$  pool in *Synechocystis* 6803 is 8 to 10-fold smaller than in *Synechococcus* 7942 (10), a smaller  $\Delta\mu\text{Na}^+$  would suffice. Measurements of  $^{22}\text{Na}^+$  uptake in *Synechococcus* 7942 showed large enhancement by the presence of  $\text{HCO}_3^-$  (8). On the other hand, Ritchie et al (21) concluded that the  $\text{Na}^+$  flux was not sufficient to support the rate of photosynthesis (thought to be supported solely by  $\text{HCO}_3^-$  transport). However, photosynthesis in both *Synechocystis* 6803 and *Synechococcus* 7942 is largely supported by  $\text{CO}_2$  uptake, even at high external pH.

The alternative possibility that  $\text{HCO}_3^-$  transport is energized by the  $\Delta\mu\text{Na}^+$  generated by a  $\text{Na}^+/\text{H}^+$  antiporter, secondary to  $\text{H}^+$ -ATPase (29), is unlikely. SbtA-mediated  $\text{HCO}_3^-$  transport activity was highest at pH 9.0 and lowest at pH 7.0 whereas the  $\Delta\mu\text{H}^+$  in cyanobacteria declines with rising pH. At alkaline pH such as 9.0,  $\Delta\mu\text{H}^+$  would not suffice to drive  $\text{HCO}_3^-$  uptake (8, 21). We cannot dismiss the possibility that  $\text{Na}^+$  binds to the  $\text{HCO}_3^-$  carrier and alters its kinetic parameters (7, 10). However, the fact that a  $\Delta\text{ntpJ}$  mutant was impaired in both the ability to grow under high  $\text{Na}^+$  and take up  $\text{HCO}_3^-$  lends support to the possibility that NtpJ is involved in  $\text{Na}^+$  extrusion rather than in the affinity of the  $\text{HCO}_3^-$  carrier for its substrate. This is further supported by the suggestion that NtpJ belongs to a  $\text{Na}^+$ -transporter family (<http://motif.genome.ad.jp/>) and it is homologous to a subunit of HKT1 in *Arabidopsis thaliana* that mediates  $\text{Na}^+$  transport (30). We suggest that it is most plausible that the SbtA-mediated  $\text{HCO}_3^-$  transport is energized by a primary  $\text{Na}^+$  pump. Detailed studies on NtpJ and homologues of other subunits of HKT1 are being performed to assess their role in

Na<sup>+</sup> extrusion.

*Comparative Sequence Analysis of SbtA*—All the cyanobacterial strains examined, with the exception of *P. marinus* strains, possess genes involved in CO<sub>2</sub> uptake (3). Phylogenetic analysis indicated that two types of SbtA exist in cyanobacteria; one in *Synechocystis* 6803, *Synechococcus* sp. PCC 6301 and *Synechococcus* sp. PCC 7002, and the other in *N. punctiforme* and *P. marinus* strains MED4 and MIT9313 (Fig. 5A). *Anabaena* sp. strain PCC 7120 possesses both types of SbtA. *N. punctiforme* is evolutionary very close to *Anabaena* PCC 7120. Therefore, it is likely that the second type of SbtA is located in the genomic regions of *Nostoc* yet to be revealed. We may conclude that *P. marinus* strains acquire Ci by HCO<sub>3</sub><sup>-</sup> transport and that the SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport plays a crucial role in the acquisition of Ci either when the supply of CO<sub>2</sub> is limited or in organisms such as *P. marinus* strains that do not possess a CO<sub>2</sub>-uptake system. The *Prochlorococcus* group is thought to be the most abundant photosynthetic organism on the planet (31), and is responsible for a significant fraction of CO<sub>2</sub> fixation in the oceans. The present study suggests a crucial role of the SbtA-mediated HCO<sub>3</sub><sup>-</sup> transport in the acquisition of Ci by *P. marinus* and, therefore, for carbon fixation in the oceans.

Bicarbonate transporters are the principal regulators of pH in animal cells and have a vital role in acid-base movement. The functional family of HCO<sub>3</sub><sup>-</sup> transporters includes Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers, three Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporters and K<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporter (32, 33). These transporters are much larger than SbtA and there was no similarity in amino-acid sequences between SbtA and mammalian-type HCO<sub>3</sub><sup>-</sup> transporters.

#### FOOTNOTES

\*This study was supported by: a Grant-in-Aid for Scientific Research (B) (2)(12440228); The Human Frontier Science Program (RG0051/1997M) to T.O.; a Grant-in-Aid for Scientific Research (No. 12660300) to H.F; "Research for the Future" Program (JSPS-RFTF97R16001) to T.O./H. F; USA-Israel Binational Science Foundation (BSF) to A.K. and program MARS2 a cooperation of the German BMFT and the Israeli MOST to A.K.

<sup>1</sup>The abbreviations used are: CCM, CO<sub>2</sub>-concentrating mechanism; Chl, chlorophyll; Ci, inorganic carbon; H-cells, cells grown under 3% (v/v) CO<sub>2</sub> in air; L-cells, cells acclimated to air for 18 hrs in the light; WT, wild type.

## References

1. Kaplan, A., and Reinhold, L. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 539-570.
2. Badger, M. R., and Spalding, M. H. (2000) In: *Advances in Photosynthesis*, vol. 9. *Photosynthesis: Physiology and Metabolism*, eds, Leegood, R. C., Sharkey, T. D. and von Caemmerer, S. (Kluwer Acad. Pub., Dordrecht) pp.399-434.
3. Shibata, M., Ohkawa, H., Kaneko, T., Fukuzawa, H., Tabata, S., Kaplan, A., and Ogawa, T. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 11789-11794.
4. Ohkawa, H., Pakrasi, H. B., and Ogawa, T. (2000) *J. Biol. Chem.* **275**, 31630-31634.
5. Omata, T., Price, G. D., Badger, M. R., Okamura, M., Goh,ta, S. and Ogawa, T. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 13571-13576.
6. Miller, A. G., Turpin, D. H. and Canvin, D. T. (1984) *J. Bacteriol.*, **159**, 100-106.
7. Reinhold, L., Volokita, M., Zenvirth, D., and Kaplan, A. (1984) *Plant Physiol.*, **76**, 1090-1092.
8. Kaplan, A., Lerner, M., and Scherer, S. (1989) *Plant Physiol.* **89**, 1220-1225.
9. Espie, G. S., and Kandasamy, R. A. (1994) *Plant Physiol.* **104**, 1419-1428.
10. So, A. K. C., Kassam, A., and Espie, G. S. (1998) *Can. J. Bot.* **67**, 1084-1091.
11. Stanier, R. Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. (1971) *Bacterial Rev.* **35**, 171-205.
12. Ohkawa, H., Price, G. D., Badger, M. R., and Ogawa, T. (2000) *J. Bacteriol.* **182**, 2591-2596.
13. Ogawa, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4275-4279.



14. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Research* **3**, 109-136.
15. Chelly, J., and Kahn, A. (1994) In : *The Polymerase Chain Reaction*, eds., Mullis, K.B., Ferre', F., and Gibbs, R.A. (Birkhauser, Boston) pp97-109.
16. Aiba, H., Adhya, S., and de Crombrughe, B. (1981) *J. Biol. Chem.* **256**, 11905-11910.
17. Agustin, V. (1992) *Nucleic Acids Res.* **20**, 6331-6337.
18. Soltes-Rak, E., Mulligan, M., E. and Coleman, J. R. (1997) *J. Bacteriol.* **179**, 769-774.
19. Scherer, S., Riege, H., and Boger, P. (1988) *Plant Physiol.* **86**, 939-941.
20. Katoh, A., Sonoda, M., Katoh, H., and Ogawa, T. (1996) *J. Bacteriol.* **178**, 5452-5455.
21. Ritchie, R. J., Nadolny, C., and Larkum, A. W. D. (1996) *Plant Physiol.* **112**, 1573-1584.
22. Hagemann, M., Schoor, A., Jeanjean, R., Zuther, E., and Joset, F. (1997) *J. Bacteriol.* **179**, 1727-1733.
23. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. III, Tekaiia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, S., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, S., Squares, S., Squares, R., Sulston, J.E., Taylor, K., Whitehead, S., and Barrell, B.G. (1998) *Nature* **393**, 537-544.
24. Nierman, W.C., Feldblyum, T.V., Laub, M.T., Paulsen, I.T., Nelson, K.E., Eisen, J.,

- Heidelberg, J.F., Alley, M.R.K., Ohta, N., Maddock, J.R., Potocka, I., Nelson, W.C., Newton, A., Stephens, C., Phadke, N.D., Ely, B., DeBoy, R.T., Dodson, R.J., Durkin, A.S., Gwinn, M.L., Haft, D.H., Kolonay, J.F., Smit, J., Craven, M.B., Khouri, H., Shetty, J., Berry, K., Utterback, T., Tran, K., Wolf, A., Vamathevan, J., Ermolaeva, M., White, O., Salzberg, S.L., Venter, J.C., Shapiro, L., and Fraser, C.M. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4136-4141.
25. Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hiramata, C., Nakamura, Y., Ogasawara, N., Kuhara, S., and Horikoshi, K. (2000) *Nucleic Acids Res.* **28**, 4317-4331.
  26. Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) *J Mol. Biol.* **300**, 1005-1016.
  27. Bonfil, D. J., Ronen-Tarazi, M., Sültemeyer, D., Lieman-Hurwitz, J., Schatz, D., and Kaplan, A. (1998) *FEBS Lett.* **430**, 236-240.
  28. Brown, I. I., Fadeyev, S. I., Kirik I. I., Severina I. I., and Skulachev, V. P. (1990) *FEBS Lett.* **270**, 203-206.
  29. Blumwald, E., Wolosyn, J. M., and Packer, L. (1984) *Biochim. Biophys. Res. Comm.* **122**, 452-459.
  30. Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, A., Bakker, E. P., Nakamura, T., and Schroeder, J. I. (2000) *Plant Physiol.*, **122**, 1249-1259.
  31. Partensky, F., Hess, W. R. and Vault, D. (1999) *Microbiol Mol Biol Rev.* **63**, 106-127.
  32. Romero, M. F., Hediger, M. A., Boulpaep, E. L., and Boron, W. F. (1997) *Nature* **387**, 409-413.

33. Soleimani, M., and Burnham, C. E. (2000) *Kidney Int.* **57**, 371-384.
34. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) *Methods Enzymol.* **266**, 383-402.
35. Kyte, J., and Doolittle, R.F. (1982) *J. Mol. Biol.* **157**, 105-132.

### Figure legends

**Fig. 1.** The structure of the *sbtA* (*slr1512*) region and the  $\text{Cm}^R$  cassette tags and  $\text{Hyg}^R$  cassette interrupting the genes (A), the growth of WT and mutants on agar plates (B) and of WT and  $\Delta\text{ndhD3/D4}/\text{cmpA}/\text{sbtA}$  mutant in liquid (C) at pH 9.0 under air or air enriched with 3%  $\text{CO}_2$  (v/v). (A) The positions of the  $\text{Cm}^R$  cassette in *sbtA* are 109, 155, 441 and 1056 base pairs down-stream of the initiation codon of *sbtA* for NB-10, NB-48, NB-3 and NB-9, respectively. A fragment between 98 and 142 base pairs downstream of the initiation codon of *slr1513* was replaced with a hygromycin resistance cassette ( $\text{Hyg}^R$ ). The horizontal arrows indicate the direction of the cassettes. (B) Two  $\mu\text{l}$  of cell suspensions with densities corresponding to  $\text{OD}_{730\text{nm}}$  values of 0.1 (upper rows of panels in B), 0.01 (middle rows) and 0.001 (lower rows) were spotted on agar plates containing medium BG11 buffered at pH 9.0. The plates were incubated under 3%  $\text{CO}_2$  in air (v/v) or air alone for 5 days at  $50 \mu\text{mol photons.m}^{-2}\text{s}^{-1}$ . (C) The growth of WT (triangles) and  $\Delta\text{ndhD3/D4}/\text{cmpA}/\text{sbtA}$  mutant (circles) in BG11 (pH 9.0) under 3%  $\text{CO}_2$  in air (v/v) (H, open symbols) or air (L, closed symbols).

**Fig. 2.** The uptake of  $\text{HCO}_3^-$  by the WT and various mutants (A) and by the  $\Delta\text{ndhD3/D4}/\text{cmpA}$  mutant (B). Unless otherwise stated, cells grown at 3%  $\text{CO}_2$  in air (v/v) were aerated with air overnight and were suspended in the assay buffer of pH 9 containing 15 mM NaCl and  $400 \mu\text{M HCO}_3^-$ . Cells were suspended in the assay buffer of pH 8 and 7 for j and k, respectively, and in the assay buffer of pH 9.0 in

which NaCl was replaced with KCl for g. H-cells were used for h. Cells were incubated for 15 sec either in light (a - k) or in darkness (c' - e'). Vertical bars indicate standard deviations (n = 5).

**Fig. 3. The transcript levels of *sbtA* and *cmpA* in the WT (a, b, c) and  $\Delta ndhD3/D4$  mutant (d, e, f). Transcript abundance in H-cells (a, d) or H-cells adapted to air for 2 (b, e) and 6 hrs (c, f) was determined by the RT-PCR method (15). The transcript levels of RNaseP (17) in each sample are shown as a control. The absence of contamination of DNA was confirmed by PCR without RTase reaction.**

**Fig. 4. The uptake of  $\text{HCO}_3^-$  by the  $\Delta ndhD3/D4/cmpA$  (A, B) and  $\Delta cmpA/sbtA$  (C) strains as a function of  $\text{HCO}_3^-$  (A, C) and  $\text{Na}^+$  (B) concentrations.  $\text{HCO}_3^-$  uptake was measured in the medium of pH 9.0 containing 15 mM NaCl for (A) and (C) and 15 mM KCl/400  $\mu\text{M}$   $\text{HCO}_3^-$  for (B) and various concentrations of  $\text{HCO}_3^-$  for (A) and (C) and NaCl for (B). The closed triangles in (C) indicate the values obtained for the  $\Delta ndhD3/D4/cmpA/sbtA$  mutant. Vertical bars indicate standard deviations (n = 5).  $\text{O}_2$  evolution was measured with cells suspended in BG-11 medium buffered at pH 9.0 containing 15 mM NaCl.**

**Fig. 5. Effect of inactivation of *ntpJ* in WT and in the  $\Delta ndhD3/D4$  mutant on their growth and  $\text{HCO}_3^-$  uptake activity. (A) Growth rates of the WT and  $\Delta ntpJ$  strains in BG-11 medium, pH 8.0, containing various concentrations of NaCl under aeration**

with 3% CO<sub>2</sub> in air (v/v). (B) Growth of the WT,  $\Delta ntpJ$  and  $\Delta ndhD3/D4/ntpJ$  strains on agar plates buffered at pH 9.0 under the conditions described in the legend for Fig. 1. (C) The HCO<sub>3</sub><sup>-</sup>-transport activity of low CO<sub>2</sub>-adapted cells of the  $\Delta ndhD3/D4$  and  $\Delta ndhD3/D4/ntpJ$  mutants suspended in the assay buffer of pH 9 containing 15 mM NaCl and 400  $\mu$ M HCO<sub>3</sub><sup>-</sup>. Vertical bars indicate standard deviations (n = 5).

**Fig. 6. Phylogenetic trees of SbtA (A) and NtpJ (B).** Multiple sequence alignment was performed using the CLUSTAL program (34). Syn6803, *Synechocystis* 6803; Syn6301, *Synechococcus* sp. strain PCC 6301; Syn7002, *Synechococcus* sp. strain PCC 7002; Ana, *Anabaena* sp. strain PCC 7120; Nos, *Nostoc punctiforme*; ProMED, *P. marinus* MED4; ProMIT, *P. marinus* MIT9313; Bacillus, *Bacillus halodurans*; Caulo, *Caulobacter crescentus*; Myco, *Mycobacterium tuberculosis*.

**Fig. 7. The hydropathy profiles of two types of SbtA.** The profiles were determined by the method of Kyte and Doolittle (35) using a window size of 17 amino acids.

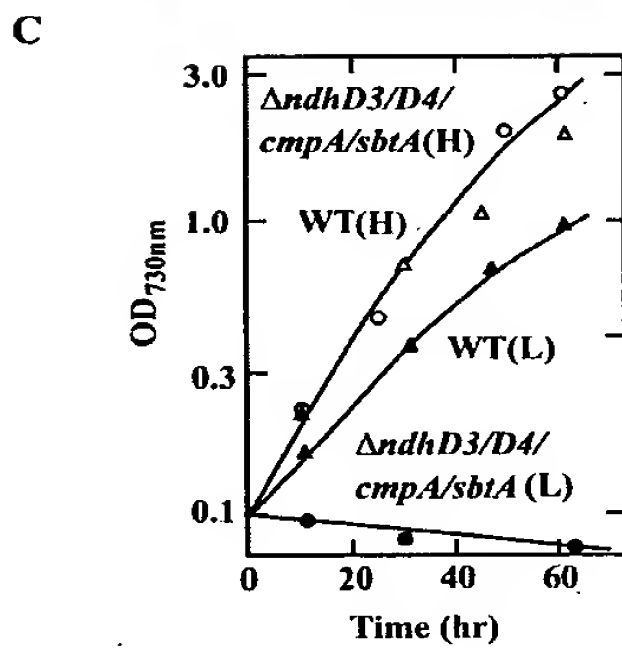
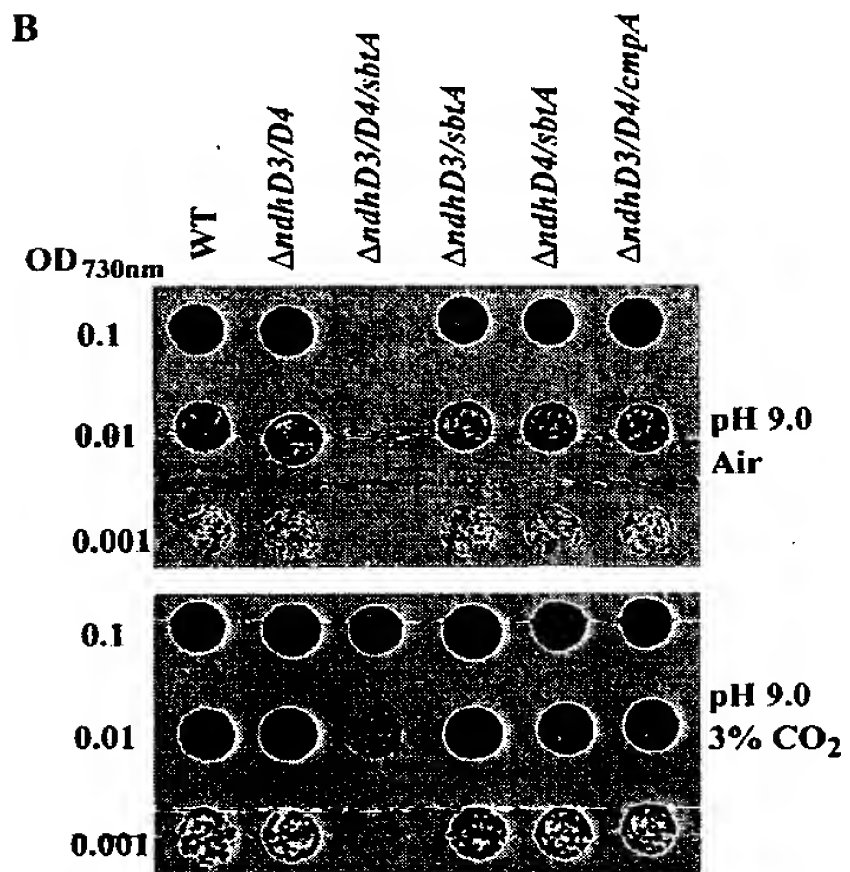
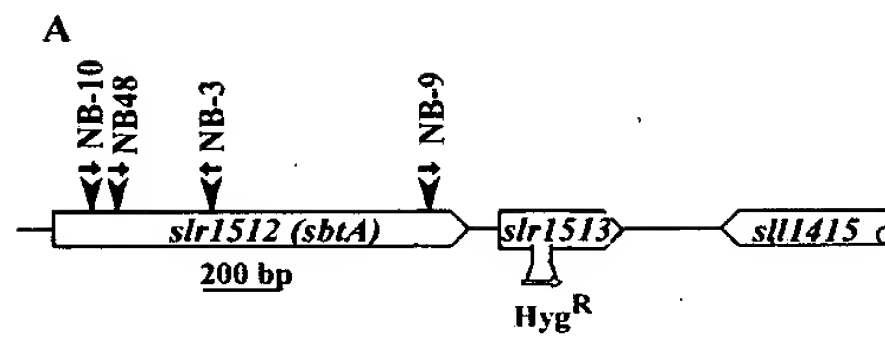


Fig. 1

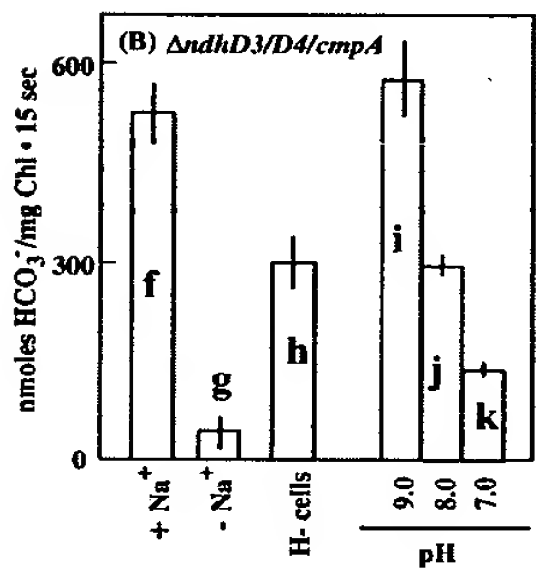
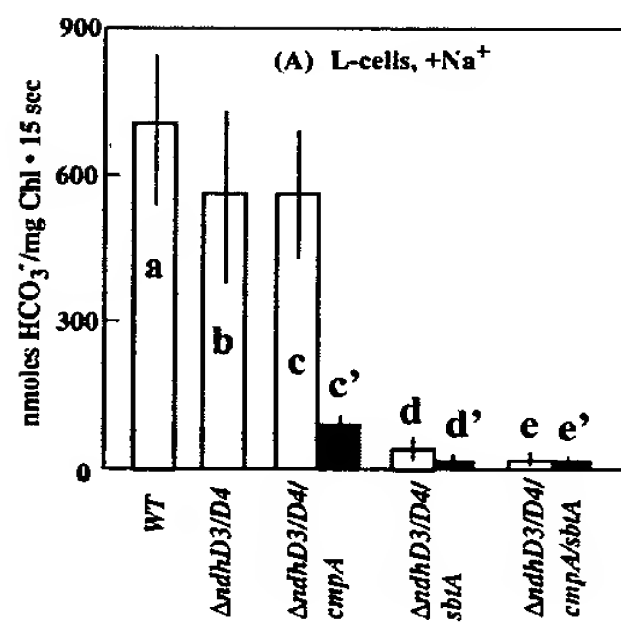


Fig.2



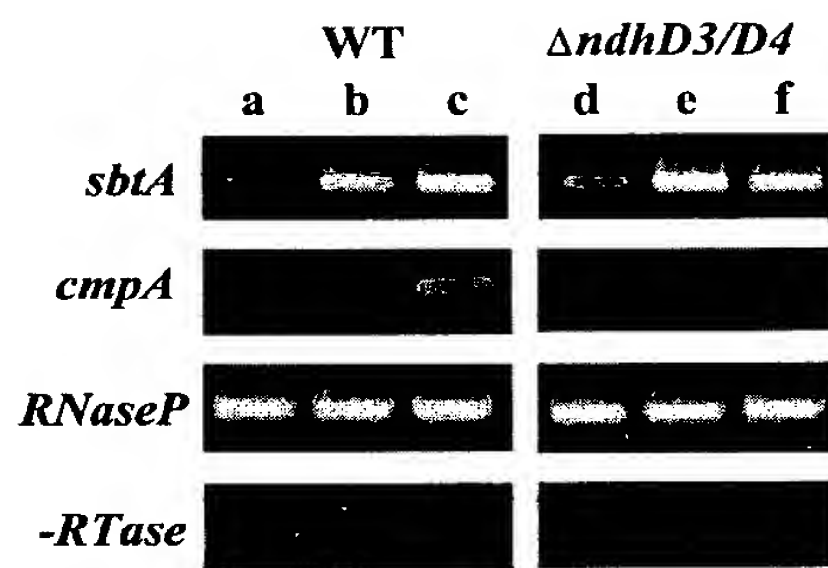


Fig.3

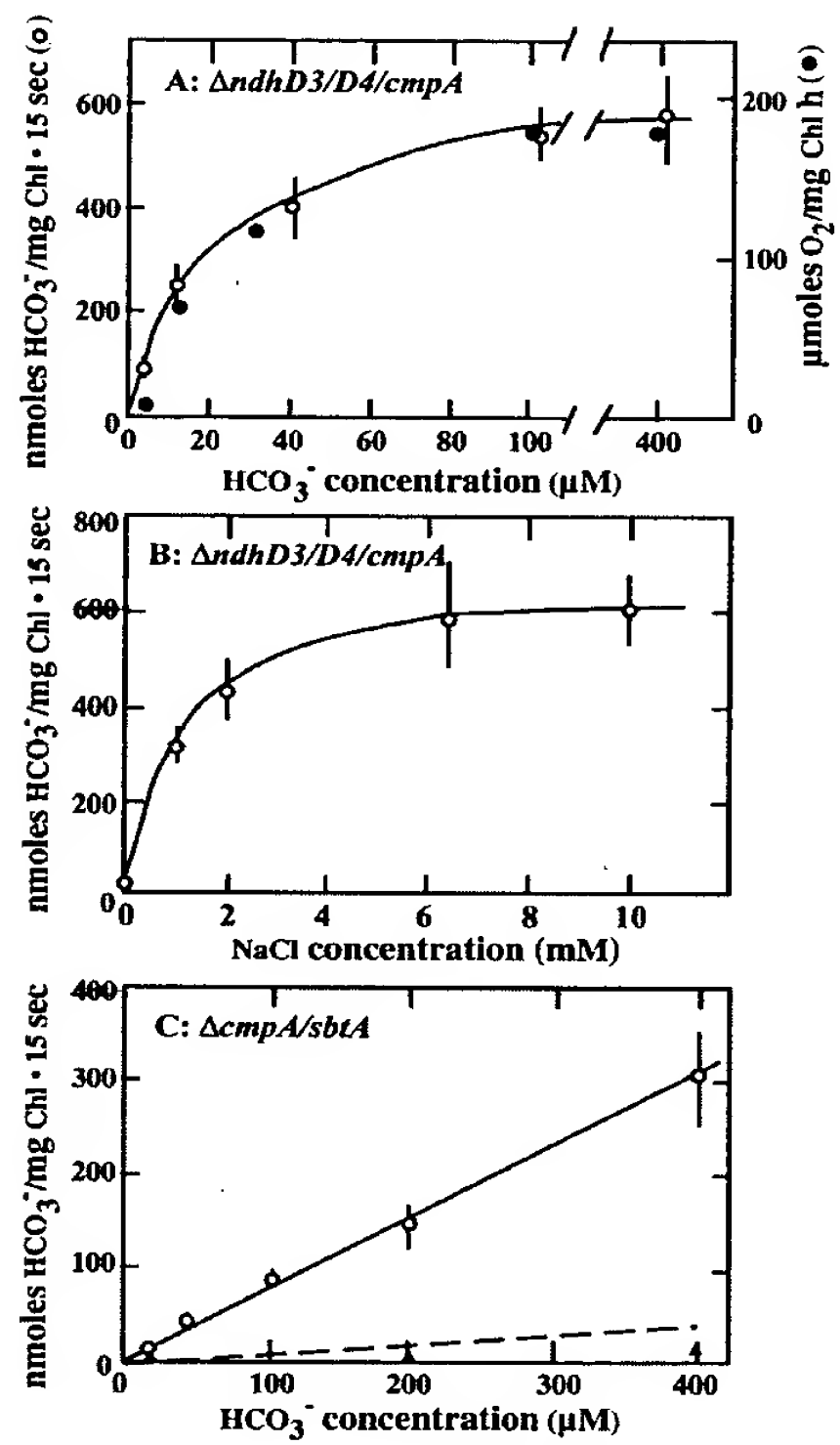


Figure 4

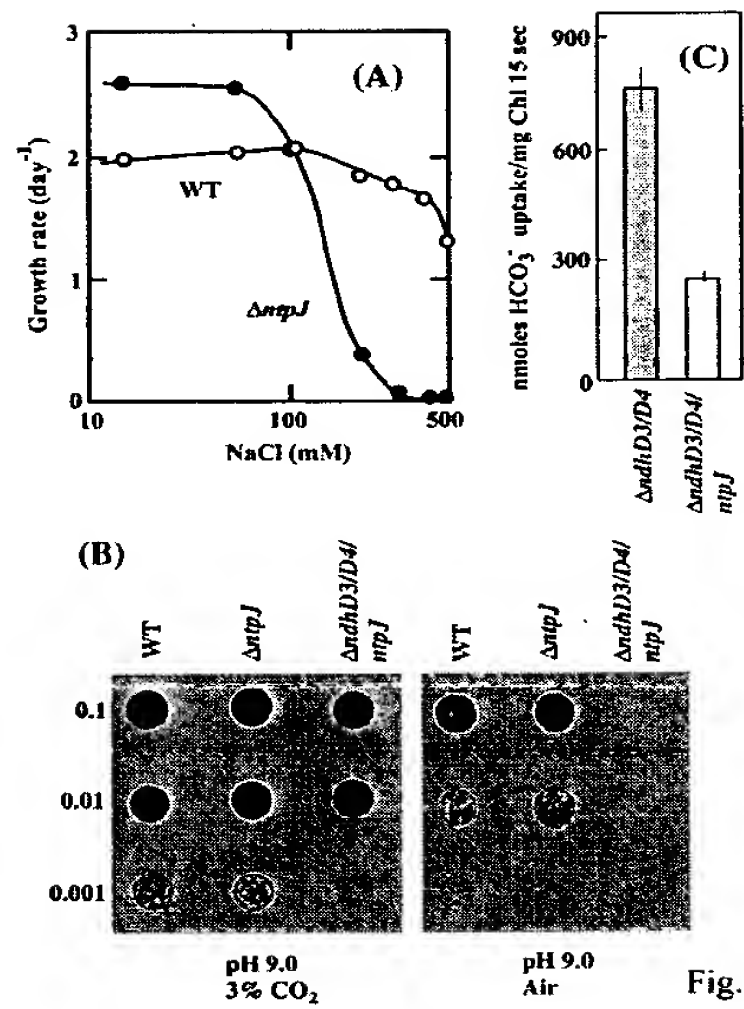


Fig.5

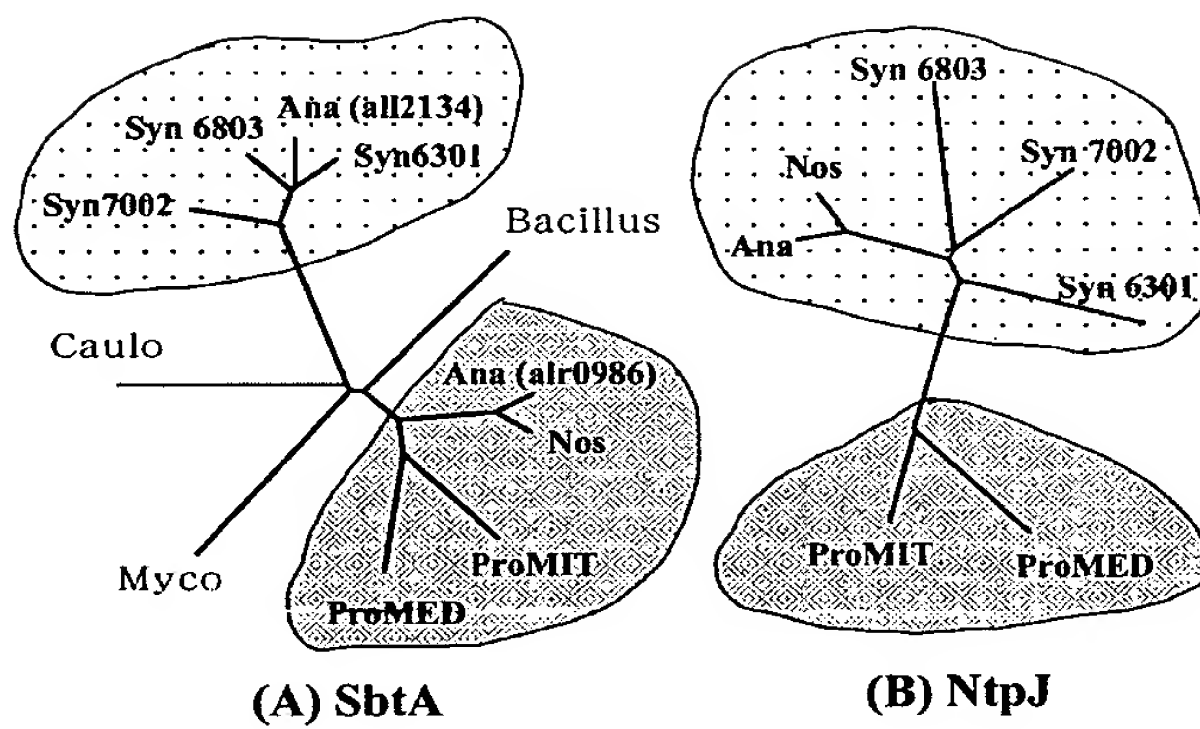


Fig. 6

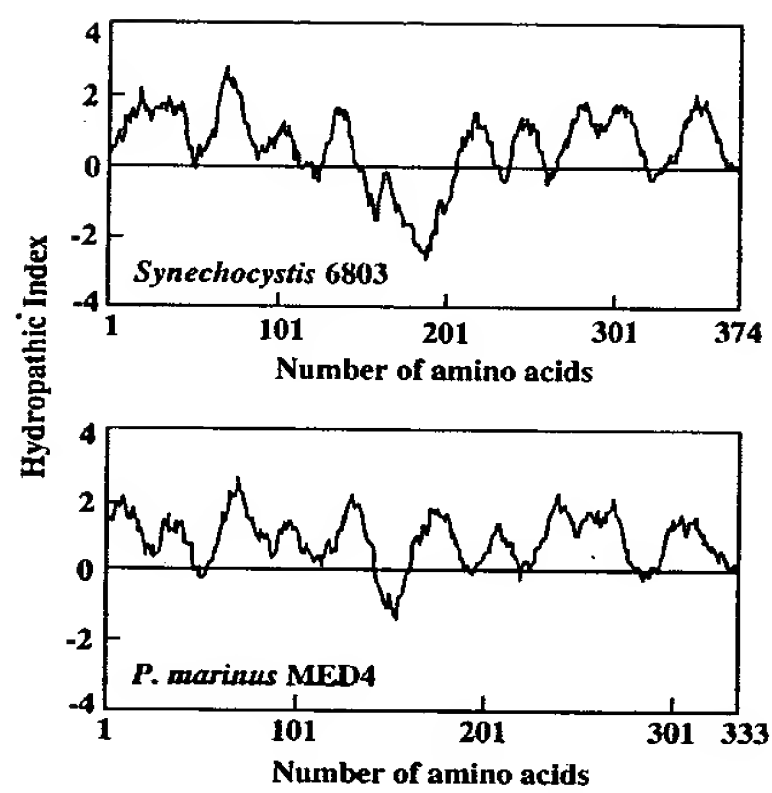


Fig. 7

# Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in $\text{HCO}_3^-$ accumulation in cyanobacteria

Judy Lieman-Hurwitz<sup>1</sup>, Shimon Rachmilevitch<sup>1</sup>, Ron Mittler<sup>2</sup>, Yehouda Marcus<sup>3</sup> and Aaron Kaplan<sup>1,\*</sup>

<sup>1</sup>Department of Plant Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel; <sup>2</sup>Department of Biology, Technion, 32000 Haifa, Israel;

<sup>3</sup>Department of Plant Sciences Tel Aviv University, 69978 Tel Aviv, Israel

Received 29 May 2002;

revised 5 September 2002;

accepted 12 September 2002.

\*Correspondence (fax 972 2 6584463;

e-mail aaronka@vms.huji.ac.il)

## Summary

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants that express *ictB*, a gene involved in  $\text{HCO}_3^-$  accumulation within the cyanobacterium *Synechococcus* sp. PCC 7942, exhibited significantly faster photosynthetic rates than the wild-types under limiting but not under saturating  $\text{CO}_2$  concentrations. Under conditions of low relative humidity, growth of the transgenic *A. thaliana* plants was considerably faster than the wild-type. This enhancement of growth was not observed under humid conditions. There was no difference in the amount of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) detected in the wild-types and their respective transgenic plants. Following activation *in vitro*, the activities of RubisCO from either low- or high-humidity-grown transgenic plants were similar to those observed in the wild-types. In contrast, the *in vivo* RubisCO activity, i.e. without prior activation, in plants grown under low humidity was considerably higher in *ictB*-expressing plants than in their wild-types. The  $\text{CO}_2$  compensation point in the transgenic plants that express *ictB* was lower than in the wild-types, suggesting that the concentration of  $\text{CO}_2$  in close proximity to RubisCO was higher. This may explain the higher activation level of RubisCO and enhanced photosynthetic activities and growth in the transgenic plants. These data indicated a potential use of *ictB* for the stimulation of crop yield.

**Keywords:** growth, *ictB*, inorganic carbon, photosynthesis, RubisCO, transgenic plants.

## Introduction

Plants that belong to the physiological  $\text{C}_4$  type or the Crassulacean acid metabolism groups, as well as many photosynthetic micro-organisms, possess various types of  $\text{CO}_2$  concentrating mechanisms (Cushman and Bohnert, 2000; Hatch, 1992; Kaplan and Reinhold, 1999). These mechanisms enable them to raise the concentration of  $\text{CO}_2$  in close proximity to ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and hence overcome, at least partly, the low affinity of the enzyme for  $\text{CO}_2$ . In contrast, the majority of higher plants that belong to the  $\text{C}_3$  group, including most crop plants, do not possess this ability. Therefore, under many environmental conditions plant photosynthesis is rate-limited by the concentration of  $\text{CO}_2$  at the carboxylation site and/or by the activity of RubisCO. Attempts are being made to raise

the apparent photosynthetic affinity of  $\text{C}_3$  plants for  $\text{CO}_2$  by various biotechnological approaches. These include a search for a RubisCO that exhibits an elevated specificity for  $\text{CO}_2$  among natural photosynthetic populations (Uemura *et al.*, 1997; Horken and Tabita, 1999; Tabita, 1999); site directed modifications of the enzyme (Cleland *et al.*, 1998; Kostov *et al.*, 1997; Ramage *et al.*, 1998; Spreitzer and Salvucci, 2002) and expression of genes involved in  $\text{C}_4$  metabolism within  $\text{C}_3$  plants (Ku *et al.*, 1999; Matsuoka *et al.*, 2001; Nomura *et al.*, 2000; Surridge, 2002). Characterization of a high- $\text{CO}_2$ -requiring mutant of the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* PCC 7942) implicated *ictB* as a gene involved in inorganic carbon accumulation in this organism (Bonfil *et al.*, 1998). *ictB* is highly conserved among cyanobacteria but its exact role is not known, since it was not possible to directly inactivate

it or its homologue *slr1515* in *Synechocystis* sp. strain PCC 6803.

Ability to stimulate photosynthesis and growth of tobacco by the expression of a single cyanobacterial gene encoding fructose-1,6/sedoheptulose-1,7-bisphosphate phosphatase was recently demonstrated (Miyagawa et al., 2001). Apparently, the level of intermediates of the Calvin cycle was raised in the transgenic tobacco plants suggesting that, under the conditions of their experiments, photosynthesis was rate-limited by the level of ribulose 1,5-bisphosphate. In this study we show that expression of *ictB* from *Synechococcus* PCC 7942 enhanced photosynthesis and growth in  $C_3$  plants due to a higher internal  $CO_2$  concentration at the site of RubisCO and consequently higher enzyme activity in the transgenic plants.

## Results

### Expression of *ictB*

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) plants bearing *ictB* were raised after transformation with *Agrobacterium* (see Experimental procedures). Northern analyses performed on total RNA isolated from the wild-type and kanamycin-resistant transformed lines demonstrated that three transgenic *Arabidopsis* (A, B and C) and five (1, 3, 4, 8 and 11) tobacco plants expressed *ictB* to different extents (Figure 1). We did not detect a transcript of *ictB* in the wild-type plants. Southern analyses of DNA from the transgenic *Arabidopsis* and tobacco plants indicated that the construct

bearing *ictB* was inserted in different sites within their genomes (not shown).

### Photosynthetic performance

In *Synechococcus* PCC 7942, *ictB* is involved in the accumulation of inorganic carbon, and a mutant impaired in this gene demanded a high  $CO_2$  concentration for photosynthesis. Therefore, we examined the rate of photosynthesis in the wild-types and the transgenic plants as it was affected by intercellular  $CO_2$  concentration. Generally, despite the fact that the expression of *ictB* varied markedly between transgenic plants, in both *Arabidopsis* and tobacco (Figure 1), there was hardly any difference between their photosynthetic performances. Plants that expressed *ictB* showed similar photosynthetic characteristics, which differed markedly from those that did not express this gene. At saturating  $CO_2$  levels, the photosynthetic rates of transgenic tobacco (Figure 2A) and *Arabidopsis* plants (Figure 2B) were similar to those found in their wild-types. This suggested that the ability to perform maximal photosynthesis was not affected by the expression of *ictB*. In contrast, under limiting intercellular  $CO_2$  concentrations, the transgenic tobacco lines 1, 3 and 11 (Figure 2A) and *Arabidopsis* plants A, B (Figure 2B) and C (not shown) showed significantly higher photosynthetic rates than the wild-types. Notably, some of the transgenic, kanamycin-resistant plants which did not express *ictB* (cf. tobacco plant number 6, Figure 2A), exhibited either similar or sometimes even slightly lower photosynthetic rates than the respective wild-type. Stomatal conductances, measured by the Li-Cor 6400 or the Delta-T porometer (model MK3, UK), were lower in plants grown under the dry conditions but did not differ significantly between the wild-types and the transgenic plants (Table 1). These data confirmed that the higher photosynthetic rate at limiting intercellular  $CO_2$

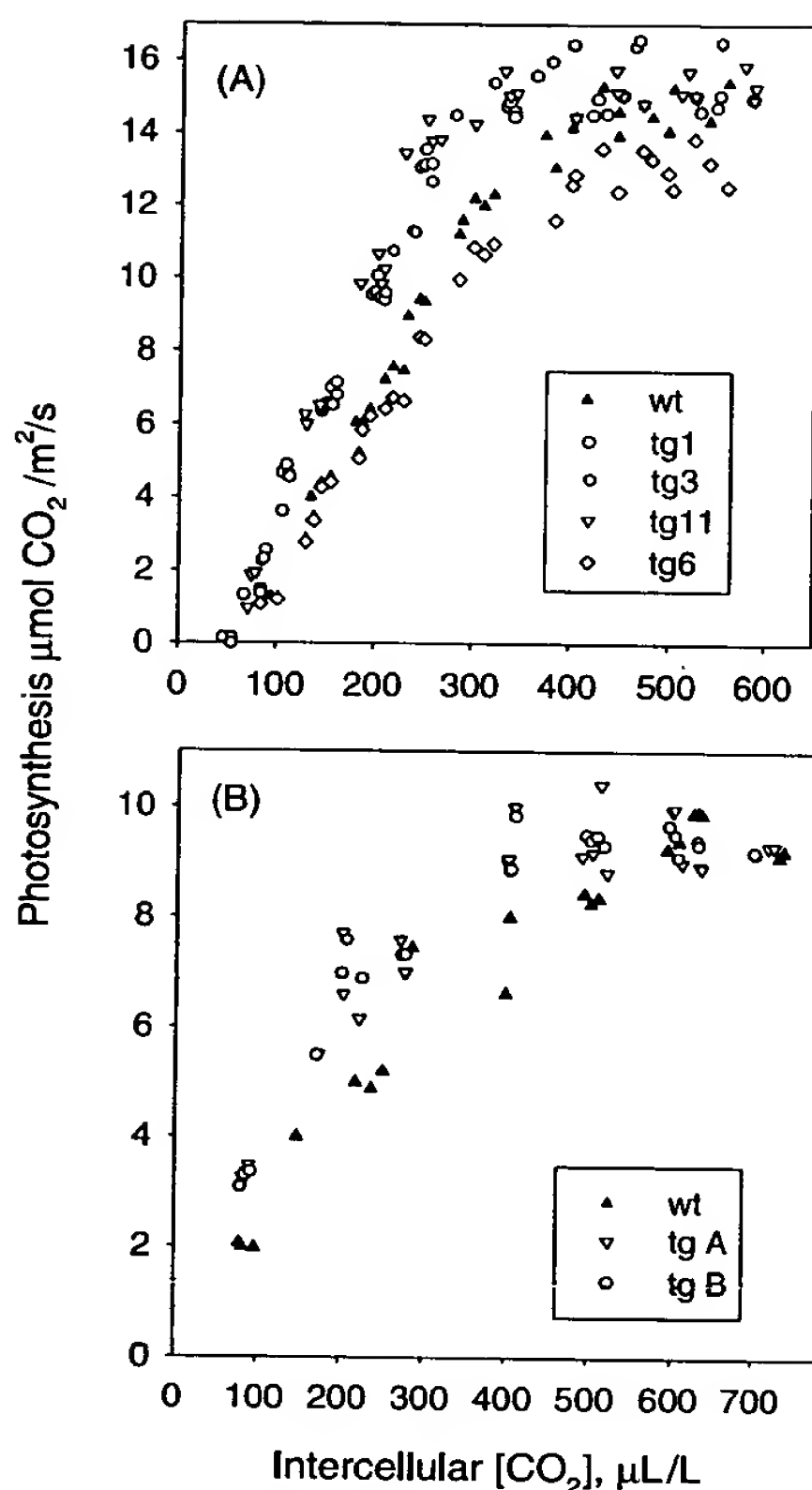


**Figure 1** Northern blots of RNA isolated from transgenic and wild-type (W) *Arabidopsis* and tobacco plants hybridized to both *ictB* and 18S rDNA probes. 30 µg RNA was loaded in each lane. RNA was isolated as described in the Experimental procedures. Northern blot analyses on 1% agarose gels, was done as described by Mittler and Zilinskas (1992).

**Table 1** Stomatal conductance in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. Plants grown under humid (70–75% relative humidity) or dry (25–30% humidity) conditions were used in these experiments

Plant	High humidity	Low humidity
Tobacco WT	686.8 ± 3.6	196.0 ± 1.2
Tobacco Plant 3	682.6 ± 4.5	196.7 ± 1.6
Tobacco Plant 11	684.3 ± 3.1	196.2 ± 1.2
<i>Arabidopsis</i> WT	597.9 ± 3.5	209.1 ± 1.3
<i>Arabidopsis</i> Plant A	598.4 ± 3.1	209.7 ± 1.7
<i>Arabidopsis</i> Plant B	599.5 ± 3.2	208.9 ± 1.3

The data are presented in mmole/m<sup>2</sup>/s, as the average ± SE, *n* = 18.



**Figure 2** The rate of photosynthesis as affected by the intercellular concentration of CO<sub>2</sub> in wild-type (wt) and transgenic (tg) tobacco (A) and *Arabidopsis* (B) plants. The plants were grown under dry conditions.  $n = 8$ .

concentrations did not result from a higher conductance of CO<sub>2</sub> in the transgenic plants.

#### CO<sub>2</sub> compensation point

Involvement of *lctB* in the ability of *Synechococcus* PCC 7942 to accumulate HCO<sub>3</sub><sup>-</sup> internally (Bonfil *et al.*, 1998) raised the possibility that the higher photosynthetic and RubisCO activities (see below) at limiting CO<sub>2</sub> concentrations in the transgenic plants were due to an elevated CO<sub>2</sub> concentration in close proximity to RubisCO. Should this be the case, it would

**Table 2** The CO<sub>2</sub> compensation points in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. The compensation points were deduced from measurements of the rate of CO<sub>2</sub> exchange over a range of CO<sub>2</sub> concentrations of 0–150 μL/L as described in Experimental procedures

Plant	CO <sub>2</sub> compensation (μL/L)
<i>Arabidopsis</i> Plant A	39.2 ± 1.0
<i>Arabidopsis</i> Plant B	41.0 ± 1.1
<i>Arabidopsis</i> WT	46.1 ± 1.1
Tobacco Plant 3	47.1 ± 1.4
Tobacco Plant 11	48.0 ± 1.6
Tobacco Wild-type	56.9 ± 1.6

The data are presented as the average ± SE,  $n = 18$ .

be expected to lower the CO<sub>2</sub> compensation point (i.e. the ambient CO<sub>2</sub> concentration, where the net CO<sub>2</sub> exchange is zero since CO<sub>2</sub> uptake in photosynthesis is equal to the sum of respiratory and photorespiratory CO<sub>2</sub> efflux). We examined the CO<sub>2</sub> compensation point in wild-type and transgenic *Arabidopsis* and tobacco plants by measuring CO<sub>2</sub> exchange in plants exposed to a range of CO<sub>2</sub> concentrations between 0 and 150 μL/L CO<sub>2</sub>. In Table 2 we show that the average CO<sub>2</sub> compensation point was significantly ( $P < 0.01$ ) lower in transgenic *Arabidopsis* and tobacco plants than in the respective wild-types. These data suggested that in both species the CO<sub>2</sub> concentration in close proximity to RubisCO was higher in the transgenic than in the wild-type plants. This is in agreement with the steeper initial slope of the curves relating CO<sub>2</sub> fixation to its concentration in the transgenic plants which express *lctB* than in the respective wild-types (Figure 2).

#### Activation state of RubisCO

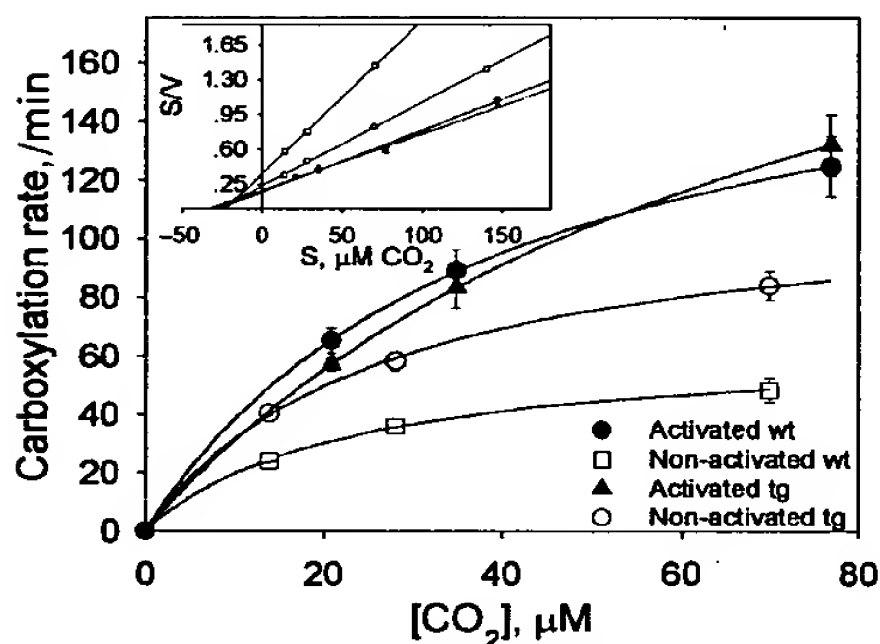
The slope of the curves relating photosynthetic rate to intercellular CO<sub>2</sub> concentration (Figure 2) was steeper in the *lctB*-expressing plants than in their corresponding wild-types, suggesting an apparent higher affinity to CO<sub>2</sub> in the transgenic plants. This could be due to a higher level of RubisCO activity (Bainbridge *et al.*, 2000; Mott and Woodrow, 2000; Poolman *et al.*, 2000). We did not detect significant differences in the abundance of active sites of RubisCO per leaf surface area or per soluble proteins between the wild-types (tobacco and *Arabidopsis*) and their respective *lctB*-expressing plants. To examine the possibility that RubisCO activity (per active site) was higher in the transgenic plants, we exposed the neighbouring leaves of wild-types and of transgenic plants, of similar age, to identical ambient conditions of light intensity



**Table 3** Rubisco activity in wild-type (WT) and transgenic tobacco plant number 3 grown in the high (70–75%) humidity chamber. Rubisco activity was determined with (*in vitro*) or without (*in vivo*) prior activation. The reaction was terminated after 1 min. Other conditions as described in Experimental procedures

Plant	Rubisco activity (nmol C fixed/nmol catalytic site/min)
WT, <i>in vitro</i>	105 ± 7
Transgenic, <i>in vitro</i>	103 ± 8
WT, <i>in vivo</i>	84 ± 7
Transgenic, <i>in vivo</i>	86 ± 6

*n* = 6, ± SD.



**Figure 3** Rubisco activity *in vivo* (non-activated) and following *in vitro* activation in the wild-type (wt) and transgenic (tg) tobacco plant 3 grown under low humidity as affected by the CO<sub>2</sub> concentration during the assay. Rate of carboxylation is in nmol CO<sub>2</sub> fixed/nmol active sites/min. The inset provides the S/V vs. S plots of data from similar experiments. *n* = 6.

and orientation, temperature and relative humidity (either high or low) for several days. The leaves were excised 3 h after onset of illumination in the growth chamber and placed in liquid nitrogen (see Experimental procedures). These experiments were performed on wild-types and transgenic *Arabidopsis* and tobacco plants. As an example, we provide detailed results obtained in the experiments with the wild-type and transgenic line 3 of tobacco (Table 3, Figure 3).

Following *in vitro* activation by the addition of CO<sub>2</sub> and MgCl<sub>2</sub>, where Rubisco activity was close to its maximum (Badger and Lorimer, 1976; Crafts-Brandner and Salvucci, 2000; Marcus and Gurevitz, 2000), there was no significant difference between the activities observed in the wild-type and transgenic plants maintained in either the humid

(Table 3) or the dry conditions (Figure 3). These data confirmed that the insertion of *ictB* did not alter the intrinsic properties of Rubisco. Under the humid conditions, the Rubisco activity observed without *in vitro* activation (most likely closely resembling those *in vivo* just before the leaves were immersed in liquid nitrogen; Bainbridge et al., 2000; Crafts-Brandner and Salvucci, 2000), was about 85% of that of the *in vitro* activated enzyme in both the wild-type and transgenic plants (Table 3). In contrast, under the low humidity conditions, the *in vivo* activity of Rubisco was about 40% higher in the transgenic than in the wild-type plants over the entire range of CO<sub>2</sub> concentrations examined in the activity assays (Figure 3). In Figure 3 we show the activities of Rubisco exposed to different CO<sub>2</sub> concentrations in order to emphasize the consistency of the data, even at various CO<sub>2</sub> levels, rather than to provide a complete account of the kinetic parameters of activated and non-activated Rubisco from tobacco. Nevertheless, an analysis of the kinetic parameters from experiments similar to that depicted in Figure 3, performed with the wild-type and transgenic line 3, indicated that while the *K<sub>m</sub>*(CO<sub>2</sub>) was scarcely affected by the expression of *ictB*, the *V<sub>max</sub>* of carboxylation, *in vivo*, was significantly higher in the *ictB*-expressing plants. The higher *in vivo* Rubisco activity in the transgenic plants as compared with the wild-type (Figure 3), under the dry conditions where stomata conductance may limit CO<sub>2</sub> supply, is consistent with the steeper slope of the curve relating photosynthetic rate to intercellular CO<sub>2</sub> concentration (Figure 2). Naturally, the *in vivo* Rubisco activities were lower than those depicted by the *in vitro* activated enzyme (Figure 3, Table 3). The reduced *in vivo* Rubisco activity in the dry vs. the high humidity grown wild-type plants is possibly due to the lower internal CO<sub>2</sub> concentration imposed by the decreased stomatal conductance. These are also the conditions where the transgenic plants exhibited faster photosynthesis (Figure 2) and growth (see below).

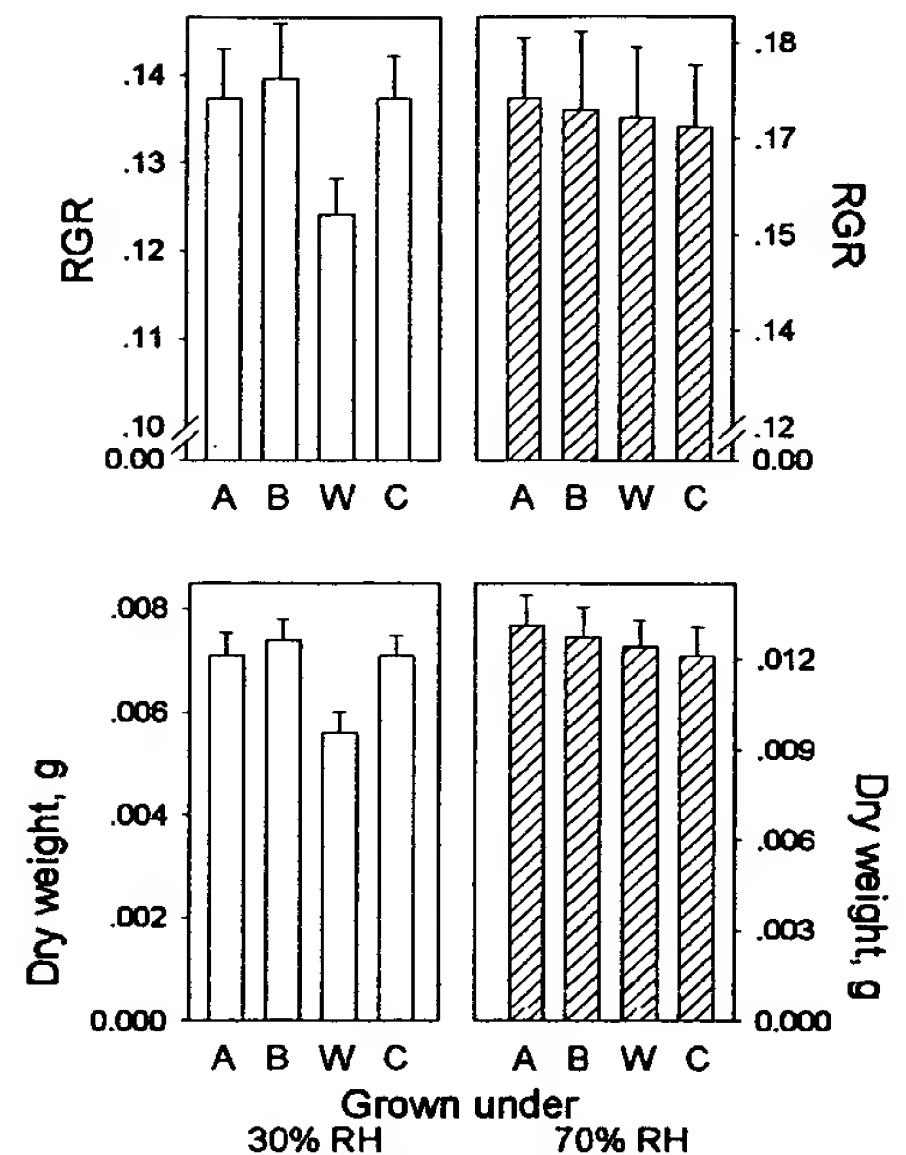
### Growth experiments

In view of the enhanced photosynthesis in the transgenic plants under CO<sub>2</sub> limiting conditions (Figure 2), we examined how their growth was affected by the relative humidity. There was no significant difference between the growth of wild-type or transgenic *Arabidopsis* plants maintained under high (70–75%) humidity (Figure 4). Under low humidity (25–30%), both wild-type and transgenic plants grew slower than in humid conditions, but the transgenic plants grew significantly faster (*P* < 0.03) than the wild-type. In Figure 4 we provide the relative growth rates (RGR) and the dry

weight accumulated over 18 days of growth. Enhancement of growth of the transgenic plants was observed throughout the growth period and not at a particular phase. Transgenic tobacco plants that expressed *ictB* also appeared to grow faster than the wild-type under low humidity. However, due to technical limitations (the size of the plants and the need to maintain them under identical conditions within the growth chamber), detailed growth experiments with enough plants to enable statistical analyses, were only performed on *Arabidopsis*.

## Discussion

Expression of a single gene from the cyanobacterium *Synechococcus* PCC 7942, *ictB*, enhanced photosynthesis and growth in  $C_3$  plants. The increased photosynthetic rate at the limiting  $CO_2$  level was most probably due to a higher RubisCO activity in the transgenic plants as opposed to another report where a higher level of fructose-1,6/sedoheptulose-1,7-bisphosphate phosphatase raised the level of ribulose 1,5-bisphosphate and thereby stimulated photosynthesis (Miyagawa *et al.*, 2001). In the absence of an independent method for directly determining  $CO_2$  concentration in close proximity to RubisCO, we had to rely on measurements of the  $CO_2$  compensation point (Table 2). The lower compensation point in transgenic plants expressing *ictB* suggested that the  $CO_2$  concentration at RubisCO sites was higher than in the wild-types, but the mechanism involved is not yet known. As indicated (Introduction), while clearly involved in  $C_i$  accumulation in *Synechococcus* PCC 7942, the role of *ictB* in  $C_i$  uptake in cyanobacteria is not yet understood. Complete inhibition of  $C_i$  transport in a *Synechocystis* sp. strain PCC 6803 mutant which possesses a normal *slr1515* (a homologue of *ictB*), suggested that *slr1515* may not be essential for  $C_i$  transport in this organism (Shibata *et al.*, 2002) which accumulates far less  $C_i$  internally than does *Synechococcus* PCC 7942. Nevertheless, it is most likely that the elevated activity of RubisCO in the transgenic plants was due to a higher  $CO_2$  concentration that could enhance the enzyme activity, both as an activator and as a substrate. Apart from the concentration of  $CO_2$ , *in vivo* RubisCO activity is affected by several effectors and parameters including light intensity, pH, the levels of specific metabolites, magnesium concentrations and the activity of RubisCO activase (Badger and Lorimer, 1976; Bainbridge *et al.*, 2000; Cleland *et al.*, 1998; Crafts-Brandner and Salvucci, 2000; Uemura *et al.*, 1997; Harrison *et al.*, 2001; Kallis *et al.*, 2000; Spreitzer, 1999). At this time it is not known whether any of these was affected by the expression of *ictB* in the transgenic plants and



**Figure 4** Growth of transgenic (A, B and C) and WT (W) *Arabidopsis* plants. Data are provided as the relative growth rate (RGR) and the average dry weight  $\pm$  SD. The growth experiments were performed six times for 18 days each,  $n = 18$ . RH, relative humidity.

thus the reason(s) for the elevated activity of their RubisCO under low humidity and limiting  $CO_2$  is not fully understood.

The level of *ictB* expression varied markedly between transgenic plants (Figure 1). Nevertheless, we did not detect statistically significant differences between *ictB*-expressing lines, within a given species, with respect to the  $CO_2$  compensation point, RubisCO activity, photosynthetic performance or growth. It is possible that despite the different levels of transcription, the abundance of IctB was similar in the transgenic plants, or that a small level of IctB suffices. Our attempts to produce reliable antibodies to IctB to examine these possibilities were not successful. Those produced, directed to the hydrophilic region within this very hydrophobic protein, were not specific enough. Furthermore, in the absence of a reliable antibody, we could not examine the location of this protein in the transgenic plants and confirm that, as expected, the fusion to the transit peptide of the small subunit of RubisCO directed the protein to the chloroplast. Nevertheless, our data clearly suggested a potential use of *ictB* in raising the yield of  $C_3$  plants, particularly under dry conditions where stomatal

closure may impose a CO<sub>2</sub> limitation and thus photosynthetic retardation.

## Experimental procedures

### Growth conditions and construction of transgenic plants

The plants used here were grown in controlled growth chambers (Binder, Germany). Tobacco (*Nicotiana tabacum*) plants were grown at 24 °C, light intensity was 350 µmol.photons/m<sup>2</sup>/s, 12 h:12 h light : dark; *Arabidopsis thaliana* plants were grown at 21 °C, 200 µmol.photons/m<sup>2</sup>/s, 8 h:16 h light : dark. The plants were grown in two growth chambers, the relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Transgenic tobacco and *Arabidopsis* expressing *ictB* were raised using a construct consisting of the 35S promoter fused to a DNA fragment encoding the transit peptide of the small subunit of RubisCO from pea, connected in-frame to *ictB*. This construct was inserted in *Agrobacterium* strains GV 3101 or LBA 4404 for *Arabidopsis* or tobacco transformations, respectively (Clough and Bent, 1998; Fraley et al., 1985). The *Agrobacterium* vectors contained a NOS terminator and a kanamycin-resistance encoding cassette.

### Measurements of photosynthetic rate and CO<sub>2</sub> compensation point

CO<sub>2</sub> and water vapour exchange were determined with the aid of a Li-Cor 6400, operated according to the manufacturer's instructions (Li-Cor, Lincoln, NE). Saturating light intensities of 750 and 500 µmol.photons/m<sup>2</sup>/s were used during the measurements with tobacco and *Arabidopsis*, respectively. The CO<sub>2</sub> compensation point was deduced from measurements of the rate of CO<sub>2</sub> exchange as affected by a range (0–150 µmole CO<sub>2</sub>/L) of CO<sub>2</sub> concentrations. The CO<sub>2</sub> concentration where the curve relating net CO<sub>2</sub> exchange to concentration crossed zero CO<sub>2</sub> was taken as the compensation point.

### Measurements of RubisCO activity

The plants were grown for 18 days under low or high relative humidity with temperature and light conditions as above. They were placed at a similar distance and orientation from the light sources to minimize possible differences between them due to unequal local conditions. The leaves were excised 3 h after the onset of illumination and immersed immediately in liquid nitrogen. Fifteen cm<sup>2</sup> of frozen leaves

were ground in a buffer containing 1.5% PVP, 0.1% BSA, 1 mM DTT, protease inhibitors (Sigma) and 50 mM HEPES-NaOH pH 8.0. For *in vitro* activation, the extracts were centrifuged and aliquots of the supernatants were supplemented with 10 mM NaHCO<sub>3</sub> and 5 mM MgCl<sub>2</sub> (Badger and Lorimer, 1976) and maintained for at least 20 min at 25 °C. RubisCO activity was determined, either immediately or after the activation (Marcus and Gurevitz, 2000) in the presence of 20–150 µM <sup>14</sup>CO<sub>2</sub> (6.2–9.3 Bq/nmole). The reaction was terminated after 1 min by 6 M acetic acid and the acid-stable products were counted in a scintillation counter (Marcus and Gurevitz, 2000). Time course analyses indicated that the RubisCO activities were constant for 1 min and declined thereafter, probably due to the accumulation of inhibitory intermediate metabolites (Cleland et al., 1998; Edmondson et al., 1990; Kane et al., 1998). Quantification of the amount of RubisCO active sites was performed as in Marcus and Gurevitz (2000).

### Growth experiments

Wild-type and transgenic *Arabidopsis* plants were germinated and maintained for 10 days under humid conditions. To minimize possible variations in water supply between transgenic and wild-type plants, the seedlings were transferred to pots, each containing a wild-type and three different transgenic plants. Twelve pots were placed in each growth chamber (Binder, Germany) under equal light intensity and temperature. The relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Other growth conditions were as above. The plants were harvested after 18 days of growth, quickly weighed (fresh weight) and dried in the oven (dry weight). The growth experiments were repeated six times.

### RNA isolation

For each sample, 1–2 g of plant material were ground in liquid nitrogen using a mortar and pestle and then transferred to 5 mL of Tris-HCl buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM Aurin tricarboxylic acid (ATA) and 14 mM β-mercaptoethanol) at room temperature, vortexed, and incubated on ice for 10 min. After adding 0.7 mL of ice-cold 3 M KCl the homogenates were vortexed and incubated on ice for 15 min. They were then centrifuged in a Sorvall SS-34 rotor at 10 000 r.p.m. for 10 min at 4 °C. The supernatants were transferred to new tubes containing 2 mL of 8 M LiCl and well mixed. The samples were incubated overnight at 4 °C and then centrifuged as described above.

The supernatants were discarded and the tubes were left upside down to drain for two min. The pellets were dissolved by vortexing in 0.4 mL of DEPC-treated water containing 0.5 mM ATA.

The RNA from each sample was extracted with 0.6 neutralized phenol in microfuge tubes and the upper aqueous phase was transferred to a new sterile tube containing 0.1 volume of 3 M Na-acetate and vortexed. Two volumes of cold ethanol were added, vortexed and incubated at  $-20^{\circ}\text{C}$  for 2 h. The samples were centrifuged for 30 min at 16 000 *g* in a microfuge. The pellets were rinsed with 1 mL cold 80% ethanol, centrifuged 10 min at 14000 r.p.m. and then the supernatants were discarded. The pellets were air-dried for 5 min and then resuspended in 25  $\mu\text{L}$  sterile water containing 0.5 mM ATA.

## Acknowledgements

We thank Dr Leonora Reinhold for helpful discussions. The research was partly supported by a grant from the Deutsch Foundation for Applied Sciences, the Hebrew University of Jerusalem.

## References

- Badger, M.R. and Lorimer, G.H. (1976) Activation of ribulose-1,5-bisphosphate oxygenase: The role of  $\text{Mg}^{2+}$ ,  $\text{CO}_2$ , and pH. *Arch. Biochem. Biophys.* **175**, 723–729.
- Bainbridge, G., Madgwick, P., Parmar, S., Mitchell, R., Paul, M., Pitts, J., Keys, A.J. and Parry, M.A.J. (2000) Engineering Rubisco to change its catalytic properties. *J. Exp. Bot.* **46**, 1269–1276.
- Bonfil, D.J., Ronen-Tarazi, M., Sultemeyer, D., Lieman-Hurwitz, J., Schatz, D. and Kaplan, A. (1998) A putative  $\text{HCO}_3^-$  transporter in the cyanobacterium *Synechococcus* sp. Strain PCC 7942. *FEBS Lett.* **430**, 236–240.
- Cleland, W.W., Andrews, T.J., Gutteridge, S., Hartman, F.C. and Lorimer, G.H. (1998) Mechanism of Rubisco: the carbamate as general base. *Chem. Rev.* **98**, 549–561.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Crafts-Brandner, S.J. and Salvucci, M.E. (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and  $\text{CO}_2$ . *Proc. Natl. Acad. Sci. USA*, **97**, 13430–13435.
- Cushman, J.C. and Bohnert, H.J. (2000) Genomic approaches to plant stress tolerance. *Curr. Opin. Plant Biol.* **3**, 117–124.
- Edmondson, D.L., Badger, M.R. and Andrews, T.J. (1990) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is caused by accumulation of a slow, tight-binding inhibitor at the catalytic site. *Plant Physiol.* **93**, 1390–1397.
- Fraley, R.T., Rogers, S.G., Horsch, R.B., Eichholz, D.A., Flick, J., Fink, C.L., Hoffman, N.L. and Sanders, P.R. (1985) The SEV system: a new disarmed Ti plasmid vector system for plant transformation. *Biol. Technology*, **3**, 629–635.
- Harrison, E.P., Olcer, H., Lloyd, J.C., Long, S.P. and Raines, C.A. (2001) Small decreases in SBPase cause a linear decline in the apparent RuBP regeneration rate, but do not affect Rubisco carboxylation capacity. *J. Exp. Bot.* **52**, 1779–1784.
- Hatch, M.D. (1992)  $\text{C}_4$  Photosynthesis: an unlikely process full of surprises. *Plant Cell Physiol.* **33**, 333–342.
- Horken, K.M. and Tabita, F.R. (1999) The 'green' form I ribulose 1,5-bisphosphate carboxylase oxygenase from the nonsulfur purple bacterium *Rhodobacter capsulatus*. *J. Bacteriol.* **181**, 3935–3941.
- Kallis, R.P., Ewy, R.G. and Portis, A.R. (2000) Alteration of the adenine nucleotide response and increased Rubisco activation activity of *Arabidopsis* Rubisco activase by site-directed mutagenesis. *Plant Physiol.* **123**, 1077–1086.
- Kane, H.J., Wilkin, J.M., Portis, A.R. and Andrews, T.J. (1998) Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate. *Plant Physiol.* **117**, 1059–1069.
- Kaplan, A. and Reinhold, L. (1999) The  $\text{CO}_2$  concentrating mechanisms in photosynthetic microorganisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 539–570.
- Kostov, R.V., Small, C.L. and McFadden, B.A. (1997) Mutations in a sequence near the N-terminus of the small subunit alter the  $\text{CO}_2/\text{O}_2$  specificity factor for ribulose bisphosphate carboxylase/oxygenase. *Photosynth. Res.* **54**, 127–134.
- Ku, M.S.B., Agarie, S., Nomura, M., Fukayama, H., Tsuchida, H., Ono, K., Hirose, S., Toki, S., Miyao, M. and Matsuoka, M. (1999) High-level expression of maize phosphoenolpyruvate carboxylase in transgenic rice plants. *Nature Biotechnol.* **17**, 76–80.
- Marcus, Y. and Gurevitz, M. (2000) Activation of cyanobacteria RuBP-carboxylase/oxygenase is facilitated by inorganic phosphate via two independent mechanisms. *Eur. J. Biochem.* **267**, 5995–6003.
- Matsuoka, M., Furbank, R.T., Fukayama, H. and Miyao, M. (2001) Molecular engineering of  $\text{C}_4$  photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 297–314.
- Mittler, R. and Zilinskas, B.A. (1992) Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *J. Biol. Chem.* **267**, 21802–21807.
- Miyagawa, Y., Tamoi, M. and Shigeoka, S. (2001) Overexpression of a cyanobacterial fructose-1,6/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nature Biotech.* **19**, 965–969.
- Mott, K.A. and Woodrow, I.E. (2000) Modelling the role of Rubisco activase in limiting non-steady-state photosynthesis. *J. Exp. Bot.* **51**, 399–406.
- Nomura, M., Sentoku, N., Nishimura, A., Lin, J.H., Honda, C., Taniguchi, M., Ishida, Y., Ohta, S., Komari, T., Miyao-Tokutomi, M., Kano-Murakami, Y., Tajima, S., Ku, M.S.B. and Matsuoka, M. (2000) The evolution of  $\text{C}_4$  plants: acquisition of cis-regulatory sequences in the promoter of  $\text{C}_4$ -type pyruvate, orthophosphate dikinase gene. *Plant J.* **22**, 211–221.
- Poolman, M.G., Fell, D.A. and Thomas, S. (2000) Modelling photosynthesis and its control. *J. Exp. Bot.* **51**, 319–328.
- Ramage, R.T., Read, B.A. and Tabita, F.R. (1998) Alteration of the alpha helix region of cyanobacterial ribulose 1,5-bisphosphate carboxylase/oxygenase to reflect sequences found in high substrate specificity enzymes. *Arch. Biochem. Biophys.* **349**, 81–88.
- Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., Kaplan, A. and Ogawa, T. (2002) Genes essential to

- sodium-dependent bicarbonate transport in cyanobacteria: function and phylogenetic analysis. *J. Biol. Chem.* **277**, 18658–18664.
- Spreitzer, R.J. (1999) Questions about the complexity of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase. *Photosynth. Res.* **60**, 29–42.
- Spreitzer, R.J. and Salvucci, M.E. (2002) Rubisco: Structure, regulatory interactions, and possibilities for a better enzyme. *Annu. Rev. Plant Biol.* **53**, 449–475.
- Surridge, C. (2002) Agricultural biotech: the rice squad. *Nature*, **416**, 576–578.
- Tabita, F.R. (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a different perspective. *Photosynth. Res.* **60**, 1–28.
- Uemura, K., Anwaruzzaman, Miyachi, S. and Yokota, A. (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO<sub>2</sub> fixation. *Biochem. Biophys. Res. Commun.* **233**, 568–571.